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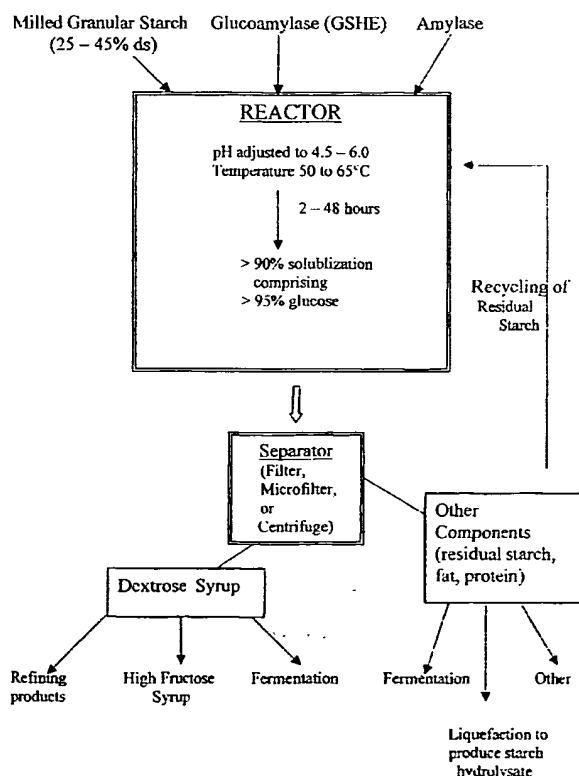
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(54) Title: EXPRESSION OF GRANULAR STARCH HYDROLYZING ENZYMES IN *TRICHODERMA* AND PROCESS FOR PRODUCING GLUCOSE FROM GRANULAR STARCH SUBSTRATES



(57) Abstract: The present invention relates to filamentous fungal host cells and particularly *Trichoderma* host cells useful for the production of heterologous granular starch hydrolyzing enzymes having glucoamylase activity (GSHE). Further the invention relates to a method for producing a glucose syrup comprising contacting a granular starch slurry obtained from a granular starch substrate simultaneously with an alpha amylase and a GSHE at a temperature equal to or below the gelatinization temperature of the granular starch to obtain a composition of a glucose syrup.

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5                   **EXPRESSION OF GRANULAR STARCH**  
                    **HYDROLYZING ENZYMES**  
                    **IN *TRICHODERMA***  
                    **AND PROCESS FOR PRODUCING GLUCOSE**  
                    **FROM GRANULAR STARCH SUBSTRATES**

10                   The present application claims priority to U.S. Provisional Patent Application Ser.  
No. 60/524,279 entitled Expression of Granular Starch Hydrolyzing Enzyme in  
*Trichoderma*, filed November 21, 2003; U.S. Provisional Patent Application Ser. No. 60/  
531,953 entitled Enzyme Compositions for Glucose Feed from Granular Starch  
15                   Substrates, filed December 22, 2003; and U.S. Provisional Patent Application Serial No.  
60/566,358 entitled Expression of Granular Starch Hydrolyzing Enzyme in *Trichoderma*,  
filed April 28, 2004.

**FIELD OF THE INVENTION**

20                   The present invention relates to filamentous fungal host cells useful for the  
expression of heterologous granular starch hydrolyzing enzymes having glucoamylase  
activity (GSHE). The invention further relates to the use of the GSHE in methods for  
producing glucose syrup and other end products from granular starch substrates  
comprising contacting a granular starch substrate, at or below the gelatinization  
25                   temperature of the granular starch, simultaneously with a starch liquefying amylase and a  
GSHE. The invention further relates to enzyme compositions comprising the GSHE and  
starch liquefying amylase.

30                   **BACKGROUND OF THE INVENTION**

                    Industrial fermentation predominately uses glucose as a feedstock for the  
production of a multitude of proteins, enzymes, amino acids, alcohols, organic acids,  
pharmaceuticals and other chemicals. In many applications, the glucose is produced from  
the enzymatic conversion of carbon substrates such as biomass and starch. Starch, which  
35                   is abundantly found in green plants, accumulates as microscopic granules varying in

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diameter from 0.5 to 175 microns. The partial crystalline nature of these starch granules imparts insolubility in cold water. As a result, the solubilization of starch granules in water requires a tremendous amount of heat energy to disrupt the crystalline structure of the granule resulting in the solubilization of partially hydrolyzed starch. Numerous  
5 solubilization processes have been employed and these include direct and indirect heating systems, such as direct heating by steam injection. (See for example, STARCH CHEMISTRY AND TECHNOLOGY, eds R. L. Whistler et al., 2<sup>nd</sup> Ed.; 1984 Academic Press Inc., Orlando, FL; STARCH CONVERSION TECHNOLOGY, Eds. G.M.A. Van Beynum et al., Food Science and Technology Series, Marcel Dekker Inc., NY; and THE ALCOHOL TEXTBOOK, 3<sup>rd</sup> Ed.,  
10 Eds. K. Jacques, T.P. Lyons and D.R. Kelsall, 1999, Nottingham University Press, UK).

In general, two enzyme steps have been used for the hydrolysis of starch to glucose. The first step is a liquefaction step, and the second step is a saccharification step. In the liquefaction step, the insoluble starch granules are slurried in water, gelatinized with heat and hydrolyzed by a thermostable alpha amylase (EC.3.2.1.1, alpha  
15 (1-4)-glucan glucanohydrolase) in the presence of added calcium to produce a mash of dextrins. The resulting mash is generally cooled to about 60 to 65°C. In the saccharification step, the soluble dextrins (sugars) are further hydrolyzed to dextrose (glucose) by an enzyme having glucoamylase (EC 3.2.1.3, alpha (1,4)-glucan  
glucohydrolase) activity. Glucose may then be used as an end product or used as a  
20 precursor to be converted into other commercially important desired end products, such as fructose, sorbitol, ethanol, lactic acid, ascorbic acid (ASA) intermediates and 1,3 propanediol.

In the late 1950s, glucoamylases derived from *Aspergillus niger* were commercialized, and these enzymes significantly improved the conversion of starch to  
25 glucose. Another significant improvement occurred in the 1970s. A thermostable alpha amylase having improved thermostability, pH stability and lower calcium dependency was derived and commercialized from *Bacillus licheniformis* (USP 3,912,590).

Further industrial processes have been adopted by the starch sweetener industry for the enzyme liquefaction process (USP 5,322,778). Some of these processes include, a  
30 low temperature process (105 -110°C for 5 - 8 min) with lower steam requirements and a high temperature process (148°C +/-5 °C for 8 -10 sec), which improves gelatinization of the starch granules resulting in improved filtration characteristics and quality of the liquefied starch substrate (Shetty, et al., (1988) *Cereal Foods World* 33:929-934).

While enzyme starch liquefaction processes are well established, improvements  
35 with respect to yield loss, processing costs, energy consumption, pH adjustments,

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temperature thresholds, calcium requirement and the levels of retrograded starch would be desirable. In particular, it is well known that residual alpha amylase from the liquefaction step, under saccharification conditions, has an adverse effect on process efficiency and that the residual alpha amylase must be inactivated prior to saccharification  
5 by glucoamylases. Inactivation is generally accomplished by lowering the pH of the liquefied starch to pH 4.2 to 4.5 at 95°C. Another disadvantage of liquefaction processes is the alkaline isomerization of reducing groups. Alkaline isomerization results in the formation of a disaccharide, maltulose (4-alpha-D-glucopyranosyl-D-fructose). Maltulose lowers the glucose yield because it is resistant to hydrolysis by glucoamylases and alpha  
10 amylases. Further, it is difficult to control the formation of reversion reaction products catalyzed by active glucoamylases at high glucose concentration. Glucoamylases from *Aspergillus niger* are generally thermostable under the typical saccharification conditions. Therefore, a substantial amount of the glucoamylase activity may remain after the saccharification reaction. Solutions to some of the problems as discussed herein have  
15 been suggested by various researchers.

For example, Leach et al (USP 4,113,509 and USP 3,922,196) disclose a process for converting granular starch (refined) into soluble hydrolyzate by incubating the granular starch with bacterial alpha amylase at a temperature below the starch gelatinization temperature. Beta amylase was then used for hydrolysis to produce high maltose syrup.

20 European Patent Application No. 0350737 A2 discloses a process for producing maltose syrup by hydrolyzing a granular (purified) starch from corn, wheat, potato and sweet potato at 60°C without the conventional liquefaction step (gelatinization followed by liquefaction at high temperature) using an alpha amylase from *Bacillus stearothermophilus*.

25 A multi-step process to convert granular (raw) starch to glucose using a glucoamylase demonstrating raw starch hydrolyzing capability has been previously described (USP 4,618,579). However, only 60% of the starch was hydrolyzed, which then resulted in an extensive recycling process.

Not only would it be advantageous to improved upon conventional processes for  
30 granular starch conversion, but also it would be desirable to provide processes resulting in increased expression and production of the enzymes used therefore. For example, glucoamylases having granular starch hydrolyzing activity with improved characteristics such as increased specific activity, different pH ranges and/or different levels of glycosylation may be particularly advantageous for use in industrial starch conversion. The

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present invention not only meets some of these needs but also results in an increase in the efficiency of producing various end products obtained from starch hydrolysis.

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## BRIEF SUMMARY OF THE INVENTION

One embodiment of the present invention concerns a one-step process for converting granular starch to glucose by hydrolyzing granular starch at or below the gelatinization temperature of the granular starch substrate, by simultaneously contacting  
10 the ungelatinized starch substrate with an endo-acting alpha amylase and a saccharifying enzyme having glucoamylase activity, and more specifically having granular starch hydrolyzing activity.

The present invention finds utility and improvement over prior art processes in at least one of the following ways a) both the alpha amylase and the glucoamylase having  
15 granular starch hydrolyzing activity are active during saccharification; b) the starch substrate is used in granular form and the starch is hydrolyzed; c) a single pH is used for solubilization and saccharification of the granular starch; d) the saccharification time period is shorter using granular starch compared to the current saccharification time using liquefied starch substrate; e) a high glucose syrup with reduced higher sugar content is  
20 obtained compared to glucose syrup obtained from liquefied starch substrate; f) glucose loss to maltulose formation is reduced; g) milliard reactions are eliminated or minimized; h) the risk of iodine positive starch polymer formation (Blue-Sac), after saccharification due to retrograded starch formation from jet cooking is lower; i) calcium addition to the starch slurry is eliminated; and j) filtration is improved because the hydrolyzed starch will not plug  
25 the filtration system. The methods and compositions encompassed by the invention offer a more economical and efficient means to produce glucose feed for industrial and specialty chemicals.

In one aspect of the invention a filamentous fungal strain transformed with a heterologous polynucleotide encoding a granular starch hydrolyzing enzyme having  
30 glucoamylase activity (GSHE) is provided. A preferred filamentous fungal strain is a *Trichoderma* strain and more specifically a *T. reesei* strain which expresses and secretes GSHE into the culture medium.

In some embodiments of this aspect, the invention pertains to a method of producing a GSHE in a filamentous host cell which comprises transforming the  
35 filamentous fungal host cell with a DNA construct comprising a promoter having

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transcriptional activity in the filamentous fungal host cell operably linked to a heterologous polynucleotide encoding a GSHE, cultivating the transformed filamentous fungal host cell in a suitable culture medium to allow expression of the GSHE and producing the GSHE. In other embodiments, the heterologous polynucleotide encoding the GSHE is derived from a strain of *Humicola grisea* or a strain of *Aspergillus awamori*. In other embodiments, the GSHE has at least 90% sequence identity to SEQ ID NO: 3 or at least 90% identity to SEQ ID NO: 6. In further embodiments, the GSHE produced by the recombinant host cell is recovered.

In a second aspect, the invention includes a fermentation broth produced from a culture of recombinant *Trichoderma reesei*, wherein the *T. reesei* comprises a heterologous polynucleotide encoding a GSHE having at least 90% sequence identity to SEQ ID NO: 3 or at least 90% sequence identity to SEQ ID NO: 6.

In a third aspect, the invention pertains to a one-step process for producing a glucose syrup from a granular starch substrate, the process comprising (a) contacting a slurry of a granular starch substrate having a dry solid content (ds) of 10 – 55% simultaneously with an alpha amylase and a granular starch hydrolyzing enzyme having glucoamylase activity (GSHE), at a temperature equal to or below the gelatinization temperature of the starch substrate, and (b) allowing the alpha amylase and the GSHE to act for a period of time sufficient to hydrolyze the granular starch to obtain a glucose syrup. In one embodiment, at least 95% of the granular starch is hydrolyzed. In a second embodiment, the yield of the glucose syrup is at least 90% by weight. In a third embodiment, the dry solid content of the granular starch substrate is between about 15 to 40%. In a fourth embodiment, the period of time to hydrolyze the granular starch is in the range of about 5 hours to 100 hours. In a fifth embodiment, the alpha amylase is an enzyme having EC 3.2.1.1. In a sixth embodiment, the alpha amylase is derived from a *Bacillus* and particularly a strain of *B. stearothermophilus*. In further embodiments, the alpha amylase is derived from a recombinant *Bacillus* strain. In a seventh embodiment, the GSHE is a glucoamylase derived from a *Humicola grisea* var. *thermoidea* strain or an *Aspergillus awamori* var. *kawachi* strain. In an eighth embodiment, the GSHE is a glucoamylase derived from a recombinant *Trichoderma* strain, and particularly a *T. reesei* strain which expresses a heterologous gene encoding a *Humicola grisea* GSHE or an *Aspergillus awamori* var. *kawachi* GSHE. In a ninth embodiment, the process further comprises separating the glucose syrup, particularly by filtration. In a tenth embodiment, the glucose from the glucose syrup is further converted to fructose. In an eleventh

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embodiment, the temperature of the one-step process is conducted at about 50 to about 70°C. In a twelfth embodiment, the pH of the process is conducted at pH 4.5 to 6.5.

In a fourth aspect, the invention relates to a one-step process for producing a glucose syrup from a granular cornstarch substrate, the process comprising (a) contacting  
5 a slurry of a granular starch substrate having a dry solid content (ds) of 25 - 45% simultaneously with an alpha amylase derived from a *Bacillus* and a glucoamylase having granular starch hydrolyzing activity which is derived from a fungal source, at a temperature of about 55 to 65°C and a pH of about 5.0 to 6.0 and allowing the alpha amylase and the glucoamylase having granular starch hydrolyzing activity to act for a period of time  
10 sufficient to hydrolyze the granular starch to obtain a glucose syrup. In one embodiment, at least 80% of the granular starch is hydrolyzed and the yield of glucose syrup is at least 90% by weight. In a second embodiment, the glucoamylase is a GSHE derived from a recombinant *Trichoderma reesei* which expresses a heterologous polynucleotide encoding a *Humicola grisea* GSHE or an *Aspergillus awamori* var. *kawachi* GSHE.

In a fifth aspect, the invention relates to a method a hydrolyzing granular starch comprising contacting a slurry of granular starch having a dry solid content of 20 – 55% simultaneously with an alpha amylase and a glucoamylase having granular starch hydrolyzing activity obtained from a *Trichoderma* strain comprising a heterologous polynucleotide encoding a GSHE derived from *Humicola grisea* and allowing the alpha  
20 amylase and glucoamylase to act for a period of time sufficient to hydrolyze the granular starch. In one embodiment, at least 90% of the granular starch is hydrolyzed. In a second embodiment, the granular starch is cornstarch or wheat starch. In a third embodiment, the GSHE is provided to the slurry at a concentration of between about 0.5 to 1.0 GSHE units of *Humicola* GA/g starch; the alpha amylase is provided to the slurry at a concentration of  
25 between about 0.1 to 0.5 kg/MT of starch, the pH of the slurry is adjusted to about pH 4.5 to 6.0; and the temperature of the slurry is adjusted to about 55 to 65°C.

In a sixth aspect, the invention relates to a method for producing a glucose syrup comprising contacting a granular starch substrate simultaneously with an alpha amylase and a granular starch hydrolyzing enzyme (GSHE), wherein the GSHE is secreted from a  
30 filamentous fungal strain, said fungal strain comprising, a heterologous polynucleotide encoding a GSHE derived from a *Humicola* strain and having the amino acid sequence of at least 90% identity to SEQ ID NO: 3 to obtain a glucose syrup. In one embodiment, the glucose is further converted to a desired end product.

In a seventh aspect, the invention relates to an enzymatic composition comprising  
35 an alpha amylase and a glucoamylase having granular starch hydrolyzing activity. In one



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embodiment the alpha amylase is derived from a *Bacillus* sp. and the GSHE is derived from a *Humicola grisea* GSHE. In a second embodiment, the GSHE is derived from a *Trichoderma* strain genetically engineered to comprise a polynucleotide encoding a *Humicola grisea* GSHE. In a third embodiment, the pH of the composition is between pH 4.5 and 6.5. In a fourth embodiment, the alpha amylase is derived from a *B. stearothermophilus* strain. In further embodiment, the ratio of alpha amylase to GSHE in the enzyme composition is 15:1 to 1:15.

In an eighth aspect, the invention relates to a process for the production of a high fructose starch based syrup comprising converting the glucose syrup obtained by a method encompassed by the invention into a fructose based syrup.

In a ninth aspect, the invention relates to a method of producing an end product wherein the glucose syrup obtained by a method encompassed by the invention is subjected to fermentation. In some embodiments of this aspect, the end product is an alcohol, and preferably ethanol. In further embodiments of this aspect, the fermentation is carried out simultaneously with the contacting step and in other embodiments the fermentation is carried out separately and sequentially to the contacting step. In yet further embodiments, the fermentation product is separated from the fermentation broth.

In a tenth aspect, the invention relates to a method for producing residual starch by separating the glucose syrup produced according to the method of the invention and retaining the composition comprising residual starch. In one embodiment, the residual starch is used for the production of end products. In a second embodiment, the residual starch is recycled and simultaneously contacted with a GSHE and an alpha amylase at a temperature below the gelatinization temperature of the granular starch.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides the genomic DNA sequence coding for the native *H. grisea* var. *thermoidea* granular starch hydrolyzing enzyme having glucoamylase activity (GSHE) (SEQ ID NO: 1). The putative introns are in bold and underlined.

FIG. 2A provides the signal sequence and mature amino acid sequence for *H. grisea* var. *thermoidea* GSHE (SEQ ID NO: 2). The putative signal sequence is in bold and underlined.

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FIG. 2B provides the mature amino acid sequence for *H. grisea* var. *thermoidea* GSHE (SEQ ID NO: 3).

FIG. 3 provides an illustration of pTrex3g\_N13 plasmid, which was used for expression of the nucleic acid encoding the *Humicola grisea* GSHE and which contains the Xba1 sites flanking the fungal expression vector, wherein

- a) *cbhl* promoter is the *Trichoderma reesei* cellobiohydrolase promoter,
- b) *H. grisea gla1* is the polynucleotide encoding the *Humicola grisea* GSHE of SEQ ID NO:3,
- c) *cbhl* terminator is the *Trichoderma reesei* cellobiohydrolase terminator,
- and d) *amdS* is an *Aspergillus nidulans* acetamidase marker gene.

FIG. 4 provides the nucleotide sequence (SEQ ID NO:11) (10738 bp) of the pTrex3g\_N13 plasmid of Figure 3.

FIG. 5 provides an SDS-PAGE gel indicating the expression of *H. grisea* var. *thermoidea* GSHE in a representative fermentation run for *Trichoderma reesei* clones as described in Example 1. Lane 1 represents the commercial molecular weight marker, SeeBlue (Invitrogen); lane 2 is blank; lane 3 depicts rGSHE expression at 48 hours; lane 4 depicts rGSHE expression at 56 hours; and lane 5 depicts rGSHE expression at 64 hours.

FIG. 6 provides the genomic DNA sequence coding for the *Aspergillus awamori* var. *kawachi* GSHE (SEQ ID NO: 4). The putative introns are in bold and underlined.

FIG. 7A provides the signal sequence and mature amino acid sequence for *A. awamori* var. *kawachi* GSHE (SEQ ID NO: 5). The signal sequence is in bold and underlined.

FIG. 7B provides the mature amino acid sequence for *Aspergillus awamori* var. *kawachi* GSHE (SEQ ID NO: 6).

FIGS. 8A and 8B illustrate the pH stability as % residual activity for the native *Humicola grisea* var. *thermoidea* GSHE (nGSHE) and the expressed *H. grisea* var. *thermoidea* GSHE (RGSHE) in the *T. reesei* host (SEQ ID NO: 3), as described in Example 1.

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FIG. 9 illustrates the hydrolysis of corn starch measured as mg glucose/mg protein over time for native *Humicola grisea* var. *thermoidea* GSHE and the expressed *H. grisea* var. *thermoidea* GSHE in the recombinant *Trichoderma reesei* host as described in Example 1.

FIG. 10 provides an SDS-PAGE gel indicating the expression of *Aspergillus awamori* var. *kawachi* GSHE in a representative fermentation run for *Trichoderma reesei* clones as described in Example 2. Lane 1 represents the commercial molecular weight marker, SeeBlue (Invitrogen); lane 2 depicts rGSHE expression at 162 hours, and lane 3 is a control, which depicts the untransformed *Trichoderma reesei* host at 162 hours.

FIG. 11 is a general diagram illustrating an embodiment of the inventive process for low energy glucose production from granular starch substrates.

FIG. 12 illustrates a scanning electron micrograph of a typical corn starch granule before exposure to a process of the invention (a) and scanning electron micrographs (b – d) of residual starch after exposure to the process encompassed by the invention.

## DETAILED DESCRIPTION OF THE INVENTION

In some aspects, the present invention relies on routine techniques and methods used in the field of genetic engineering and molecular biology. The following resources include descriptions of general methodology useful in accordance with the invention: Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL* (2nd Ed., 1989); Kreigler, *GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL* (1990) and Ausubel et al., Eds. *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY* (1994).

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., *DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY*, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, *THE HARPER COLLINS DICTIONARY OF BIOLOGY*, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can

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be used in the practice or testing of the present invention, the preferred methods and materials are described.

The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Numeric ranges are inclusive of the numbers defining the range.

Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole.

#### A. Definitions

As used herein the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and/or amylopectin with the formula  $(C_6H_{10}O_5)_x$ , wherein X can be any number. In particular, the term refers to any plant-based material including but not limited to grains, grasses, tubers and roots and more specifically the plants wheat, barely, corn, rye, rice, sorghum, legumes, cassava, millet, potato, sweet potato, and tapioca.

The term "granular starch" refers to uncooked (raw) starch, which has not been subject to gelatinization.

The term "starch gelatinization" means solubilization of a starch molecule to form a viscous suspension.

The term "gelatinization temperature" refers to the lowest temperature at which gelatinization of a starch substrate begins. The exact temperature depends upon the specific starch substrate and further may depend on the particular variety of plant species from which the starch is obtained and the growth conditions.

The term "DE" or "dextrose equivalent" is an industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE that is essentially 0 and D-glucose has a DE of 100.

The term "glucose syrup" refers to an aqueous composition containing glucose solids. In one embodiment, glucose syrup will include at least 90% D-glucose and in

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another embodiment glucose syrup will include at least 95% D-glucose. In some embodiments, the terms "glucose", "glucose syrup" and "dextrose" are used interchangeably.

5 The term "total sugar content" refers to the total sugar content present in a starch composition.

The term "dry solids content (ds)" refers to the total solids of a slurry (in %) on a dry weight basis.

"Brix" refers to a well known hydrometer scale for measuring the sugar content of a solution at a given temperature. The Brix scale measures the number of grams of sucrose present per 100 grams of aqueous sugar solution (the total solubilized solid content). Brix measurements are frequently made by use of a hydrometer or refractometer.

The term "starch-liquefying enzyme" refers to an enzyme that effects the fluidization of granular starch. Exemplary starch liquefying enzymes include alpha amylases (E.C. 3.2.1.1).

15 The term "amylases" refer to enzymes that catalyze the hydrolysis of starches.

The term "alpha-amylase (E.C. class 3.2.1.1)" refers to enzymes that catalyze the hydrolysis of alpha-1,4-glucosidic linkages. These enzymes have also been described as those effecting the exo or endohydrolysis of 1,4- $\alpha$ -D-glucosidic linkages in polysaccharides containing 1,4- $\alpha$ -linked D-glucose units. Another term used to describe these enzymes is glycogenase. Exemplary enzymes include alpha-1,4-glucan 4-glucanohydrolase.

25 The terms "saccharification enzyme" and "glucoamylase" used interchangeability herein refer to the amyloglucosidase class of enzymes (EC.3.2.1.3, glucoamylase, alpha-1, 4-D-glucan glucohydrolase). These are exo-acting enzymes, which release glucosyl residues from the non-reducing ends of amylose and amylopectin molecules. The enzymes also hydrolyze alpha-1, 6 and alpha -1,3 linkages although at much slower rates than alpha-1, 4 linkages.

30 The term "granular starch hydrolyzing enzyme (GSHE)" or "an enzyme having granular starch hydrolyzing activity" as used herein specifically refers to an enzyme having glucoamylase activity and having the ability to hydrolyze starch in granular form. Preferred GSHEs are those derived from filamentous fungi wherein the GSHE is endogenous or exogenous to the filamentous fungal cell. One preferred GSHE is the native GSHE derived from *Humicola grisea* var. *thermoidea*. Another preferred GSHE is derived from *Aspergillus awamori* var. *kawachi*. A particularly preferred GSHE is a recombinant GSHE, 35 that is a GSHE expressed in a host strain that has been genetically engineered to include

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a heterologous polynucleotide encoding the GSHE. In some preferred embodiments, the GSHE is expressed as an extracellular enzyme.

The term "hydrolysis of starch" refers to the cleavage of glucosidic bonds with the addition of water molecules.

5       The term "degree of polymerization (DP)" refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Examples of DP2 are the disaccharides, such as maltose and sucrose. A DP4<sup>+</sup> (>DP3) denotes polymers with a degree of polymerization of greater than 3.

10       The term "contacting" refers to the placing of the respective enzymes in sufficiently close proximity to the respective substrate to enable the enzymes to convert the substrate to the end product. Those skilled in the art will recognize that mixing solutions of the enzyme with the respective substrates can effect contacting.

15       The term "enzymatic conversion" in general refers to the modification of a substrate by enzyme action. The term as used herein also refers to the modification of a granular starch substrate by the action of an enzyme. In a preferred embodiment, the enzymatic conversion of a granular starch substrate will result in a glucose syrup.

The term "slurry" refers to an aqueous mixture containing insoluble starch granules.

20       The term "glycosylation" refers to the post-transcriptional modification of a protein by the addition of carbohydrate moieties, wherein the carbohydrate is either N-linked or O-linked resulting in a glycoprotein. An N-linked carbohydrate moiety of a glycoprotein is attached by a glycosidic bond to the  $\beta$ -amide nitrogen of an asparagine residue. An O-linked carbohydrate is attached by a glycosidic bond to a protein through the hydroxy  
25       group of a serine or a threonine residue.

30       The term "recombinant" when used with reference e.g. to a cell, nucleic acid, protein or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

35       The terms "recombinant GSHE", "recombinantly expressed GSHE" and "recombinantly produced GSHE" refer to a mature GSHE protein sequence that is produced in a host cell from a heterologous polynucleotide. The symbol "r" may be used to

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denote recombinant. The protein sequence of a rGSHE excludes a signal sequence. In one embodiment *Humicola grisea* var. *thermoidea* GSHE expressed in a strain of *Trichoderma reesei* is denoted by "rH-GSHE".

The terms "native GSHE" and "nGSHE" mean a GSHE, which was derived from a microbial host organism other than the fungal host for which recombinant GSHE expression is desired. Preferred native GSHEs are derived from a *Humicola grisea* strain or a *Aspergillus awamori* strain.

The terms "protein" and "polypeptide" are used interchangeably herein. The conventional one-letter or three-letter code for amino acid residues is used herein.

A "signal sequence" means a sequence of amino acids bound to the N-terminal portion of a protein, which facilitates the secretion of the mature form of the protein outside the cell. The definition of a signal sequence is a functional one. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

A "gene" refers to a DNA segment that is involved in producing a polypeptide and includes regions preceding and following the coding regions as well as intervening sequences (introns) between individual coding segments (exons).

The term "nucleic acid" encompasses DNA, RNA, single stranded or double stranded and chemical modifications thereof. The terms "nucleic acid" and "polynucleotide" may be used interchangeably herein. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present invention encompasses polynucleotides, which encode a particular amino acid sequence.

A "vector" refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like.

An "expression vector" as used herein means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation.

A "promoter" is a regulatory sequence that is involved in binding RNA polymerase to initiate transcription of a gene. The promoter may be an inducible promoter or a

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constitutive promoter. A preferred promoter used in the invention is *Trichoderma reesei cbh1*, which is an inducible promoter.

“Under transcriptional control” is a term well understood in the art that indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably linked to an element which contributes to the initiation of, or promotes transcription.

“Under translational control” is a term well understood in the art that indicates a regulatory process that occurs after mRNA has been formed,

As used herein when describing proteins and genes that encode them, the term for the gene is not capitalized and is italicized, e.g. the gene that encodes the *Humicola grisea* GSHE may be denoted as *gla1*. The term for the protein is generally not italicized and the first letter is capitalized, e.g. the protein encoded by the *gla1* gene may be denoted as Gla1.

The term “operably linked” refers to juxtaposition wherein the elements are in an arrangement allowing them to be functionally related. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence.

The term “selective marker” refers to a gene capable of expression in a host that allows for ease of selection of those hosts containing an introduced nucleic acid or vector. Examples of selectable markers include but are not limited to antimicrobials (e.g. hygromycin, bleomycin, or chloramphenicol) or genes that confer a metabolic advantage, such as a nutritional advantage on the host cell.

The term “derived” encompasses the terms “originated from”, “obtained or obtainable from”, and “isolated from”.

“Host strain” or “host cell” means a suitable host for an expression vector or DNA construct comprising a polynucleotide encoding a GSHE according to the invention. Specifically, host strains are filamentous fungal cells. In a preferred embodiment of the invention, “host cell” means both the cells and protoplasts created from the cells of a filamentous fungal strain and particularly a *Trichoderma sp.* or an *Aspergillus sp.*

The term “filamentous fungi” refers to all filamentous forms of the subdivision Eumycotina (See, Alexopoulos, C. J. (1962), *INTRODUCTORY MYCOLOGY*, New York: Wiley). These fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, cellulose, and other complex polysaccharides. The filamentous fungi of the present invention are morphologically, physiologically, and genetically distinct from yeasts. Vegetative growth by filamentous fungi is by hyphal elongation and carbon catabolism is obligatory aerobic. In the present invention, the filamentous fungal parent cell may be a



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cell of a species of, but not limited to, *Trichoderma*, e.g., *Trichoderma reesei* (previously classified as *T.longibrachiatum* and currently also known as *Hypocrea jecorina*), *Trichoderma viride*, *Trichoderma koningii*, *Trichoderma harzianum*; *Penicillium* sp.; *Humicola* sp., including *Humicola insolens* and *Humicola grisea*; *Chrysosporium* sp., including *C. lucknowense*; *Gliocladium* sp.; *Aspergillus* sp., including *A. oryzae*, *A. nidulans*, *A. niger*, and *A. awamori*; *Fusarium* sp., *Neurospora* sp., *Hypocrea* sp., and *Emericella* sp. Reference is also made to Innis et al., (1985) *Sci.* 228:21 -26.

As used herein, the term "*Trichoderma*" or "*Trichoderma* sp." refer to any fungal strain, which had previously been classified as *Trichoderma* or is currently classified as *Trichoderma*.

The term "culturing" refers to growing a population of microbial cells under suitable conditions in a liquid or solid medium. In one embodiment, culturing refers to fermentative bioconversion of a granular starch substrate to glucose syrup or other desired end products (typically in a vessel or reactor).

The term "heterologous" or "exogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell. In some embodiments, the protein is a commercially important industrial protein. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes and/or synthetic genes. The term "homologous" or "endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

The terms "recovered", "isolated", and "separated" as used herein refer to a molecule, protein, cell, nucleic acid, amino acid, or carbohydrate that is removed from at least one component with which it is naturally associated.

As used herein, the terms "transformed", "stably transformed" or "transgenic" with reference to a cell means the cell has a non-native (heterologous) nucleic acid sequence integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example,

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chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

As used herein the term "specific activity" means an enzyme unit defined as the number of moles of substrate converted to product by an enzyme preparation per unit time  
5 under specific conditions. Specific activity is expressed as units (U)/mg of protein.

As used herein "enzyme activity" refers to the action of an enzyme on its substrate.

As used herein the term "enzyme unit" refers to the amount of enzyme that converts 1 mg of substrate per minute to the substrate product at optimum assay conditions. For example, in one embodiment, the term granular starch hydrolyzing enzyme  
10 unit (GSHE U) is defined as being the amount of GSHE required to produce 1 mg of glucose per minute from granular starch under assay conditions of, for example 50°C at pH 4.5. For example, in one embodiment, the term alpha amylase enzyme unit (AU) is defined as the amount of alpha amylase which hydrolyzes 1 micromole of starch substrate in 1 min under standard assay conditions of pH 5.2 and 40°C.

15 The terms "end product" or "desired end-product" refer to any carbon-source derived molecule product which is enzymatically converted from the granular starch substrate. Preferably, the end product is glucose or a glucose syrup. Glucose may then be used as a precursor for other desired end-products.

The term "residual starch" as used herein refers to the by-product or remaining  
20 components of the inventive granular starch hydrolysis process when the composition comprising the glucose syrup or other end products is separated. The residual starch includes remaining insoluble starch, left in the composition after the separation.

A "residual starch recycling step" refers to the recycling of residual starch into a vessel or reactor, which includes a GSHE and an alpha amylase.

25 The term "yield" refers to the amount of end-product or desired end-products produced using the methods of the present invention. In some preferred embodiments, the yield is greater than that produced using methods known in the art. In some embodiments, the term refers to the volume of the end product and in other embodiment the term refers to the concentration of the end product.

30 As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol. An ethanologenic microorganism is ethanologenic by virtue of their ability to express one or more enzymes that individually or together convert sugar to ethanol.

In the present context, the term "substantially pure polypeptide" means a  
35 polypeptide preparation which contains at the most 10% by weight of other polypeptide

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material with which it is natively associated (lower percentages of other polypeptide material are preferred, e.g. at the most 8% by weight, at the most 6% by weight, at the most 5% by weight, at the most 4% at the most 3% by weight, at the most 2% by weight, at the most 1% by weight, and at the most 1/2% by weight). Thus, it is preferred that the substantially pure polypeptide is at least 92% pure, i.e. that the polypeptide constitutes at least 92% by weight of the total polypeptide material present in the preparation, and higher percentages are preferred such as at least 94% pure, at least 95% pure, at least 96% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, and at the most 99.5% pure. The polypeptides disclosed herein are preferably in a substantially pure form. In particular, it is preferred that the polypeptides disclosed herein are in "essentially pure form", i.e. that the polypeptide preparation is essentially free of other polypeptide material with which it is natively associated. This can be accomplished, for example, by preparing the polypeptide by means of well-known recombinant methods.

"ATCC" refers to American Type Culture Collection located at Manassas, VA 20108 (ATCC, [www/atcc.org](http://www/atcc.org)).

"NRRL" refers to the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (and previously known as USDA Northern Regional Research Laboratory), Peoria, ILL.

"A", "an" and "the" include plural references unless the context clearly dictates otherwise.

As used herein the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

#### B. Preferred Embodiments

##### 25 Starch Substrates –

A granular starch substrate to be processed in the methods of the invention may be obtained from any plant part including stems, grains, roots and tubers. Particularly preferred plant sources include corn; wheat; rye; sorghum; rice; millet; barley; cassava; legumes, such as beans and peas; potatoes; sweet potatoes; bananas; and tapioca. The starch may be highly refined raw starch or feedstock from starch refinery processes. Specifically contemplated starch substrates are cornstarch and wheat starch. Those of general skill in the art are well aware of available methods which may be used to prepare granular starch substrates for use in the methods encompassed by the invention. Some of these available methods include dry milling of whole cereal grains using hammer mills and roller mills and wet milling.

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Various starches are commercially available. For example, cornstarches are available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starches are available from Sigma; sweet potato starches are available from Wako Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical  
 5 Pharmaceutical Co. (Japan).

While not meant to limit the invention in any manner, Table 1 below provides a general guide to the level of starch found in some common cereal grains. As one of ordinary skill in the art is well aware the level of starch in a grain may vary depending on such factors as genotype and environment.

10

TABLE 1  
 Starch Content of Various Grains

Raw Material	Starch %
Corn	60 – 68
Wheat	60 – 65
Oats	50 – 53
Barley	55 – 65
Milo	60 – 65
Potato	10 – 25
Cassava	25 – 30
Rye	60 – 65
Rice (polished)	70 – 72
Sorghum (millet)	75 - 80

The Alcohol Textbook, 3<sup>rd</sup> Ed. K. Jacques et al., Eds. 1999, Nottingham University Press, pg. 11.

15

In some embodiments of the methods encompassed by the invention, the granular starch substrate is slurried (generally with water) and the slurry comprises i) about 10 to about 55% dry solids content ; ii) about 20 to about 50% dry solids content ; iii) about 25 to about 45% dry solids content ; iv) about 30 to about 45% dry solids content; v) about 30 to  
 20 about 40% dry solids content ; and vi) about 30 to 35% dry solids content.

#### Alpha Amylases -

In some of the embodiments encompassed by the invention, the alpha amylase is a microbial enzyme having an E.C. number, E.C. 3.2.1.1-3 and in particular E.C. 3.2.1.1.  
 25 In some embodiments, the alpha amylase is a thermostable bacterial alpha amylase.

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Suitable alpha amylases may be naturally occurring as well as recombinant and mutant alpha amylases. In particularly preferred embodiments, the alpha amylase is derived from a *Bacillus* species. Preferred *Bacillus* species include *B. subtilis*, *B. stearothermophilus*, *B. lentus*, *B. licheniformis*, *B. coagulans*, and *B. amyloliquefaciens* (USP 5,763,385; USP 5,824,532; USP 5,958,739; USP 6,008,026 and USP 6,361,809). Particularly preferred alpha amylases are derived from *Bacillus* strains *B. stearothermophilus*, *B. amyloliquefaciens* and *B. licheniformis*. Also reference is made to strains having ATCC 39709; ATCC 11945; ATCC 6598; ATCC 6634; ATCC 8480; ATCC 9945A and NCIB 8059.

Commercially available alpha amylases contemplated for use in the compositions and methods of the invention include; SPEZYME AA; SPEZYME FRED; GZYME G997 (Genencor International Inc.) and TERMAMYL 120-L, LC, SC and SUPRA (Novozyme Biotech).

As understood by those in the art, the quantity of alpha amylase used in the compositions and methods of the present invention will depend on the enzymatic activity of the alpha amylase. In general, an amount of about 0.01 to 5.0 kg of the alpha amylase is added to a metric ton (MT) of the raw material (granular starch substrate). This amount is approximately equivalent to 0.06 AU/g ds to 30 AU/g ds with a GZYME 997. In some embodiments, the alpha amylase is added in an amount about 0.05 to 5.0 kg; about 0.05 to 2.5 kg; about 0.1 to 2.5 kg; about 0.1 to 2.0 kg; about 0.1 to 1.5 kg; about 0.1 to 1.0 kg; about 0.5 to 5.0 kg and about 0.5 to 2.0 kg per metric ton. These values are approximately equal to 0.3 to 30 AU/g ds; 0.3 to 15 AU/g ds; 0.6 to 15 AU/g ds; 0.6 to 12 AU/g ds; 0.6 to 9 AU/g ds; 0.6 to 6 AU/g ds; 3 to 30 AU/g ds and also 3 to 12 AU/g ds with a GZYME 997. In further embodiments, other quantities are utilized, for example, generally an amount of between about 0.01 to 1.0 kg of GZYME 997 or SPEZYME FRED (Genencor International Inc.) is added to a metric ton of starch. In other embodiments, the enzyme is added in an amount between about 0.05 to 1.0 kg; between about 0.1 to 0.6 kg; between about 0.2 to 0.6 kg and between about 0.4 to 0.6 kg of GZYME 997 or SPEZYME FRED per metric ton of starch.

Granular starch hydrolyzing enzymes having glucoamylase activity -

Glucoamylases (E.C. 3.2.1.3) are enzymes that remove successive glucose units from the non-reducing ends of starch. The enzyme can hydrolyze both linear and branched glucosidic linkages of starch, amylose and amylopectin. While glucoamylase may be derived from bacteria, plants and fungi, preferred glucoamylases encompassed by the present are derived from fungal strains.

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Glucoamylases secreted from fungi of the genera *Aspergillus*, *Rhizopus*, *Humicola* and *Mucor* have been derived from fungal strains, including *Aspergillus niger*, *Aspergillus awamori*, *Rhizopus niveus*, *Rhizopus oryzae*, *Mucor miehe*, *Humicola grisea*, *Aspergillus shirousami* and *Humicola (Thermomyces) laniginosa* (See, Boel et al. (1984) *EMBO J.* 3:1097-1102; WO 92/00381; WO 00/04136; Chen et al., (1996) *Prot. Eng.* 9:499-505; Taylor et al., (1978) *Carbohydrate Res.* 61:301 – 308 and Jensen et al., (1988) *Can. J. Microbiol.* 34:218 – 223).

Enzymes having glucoamylase activity used commercially are produced for examples, from *Aspergillus niger* (trade name OPTIDEX L-400 and G ZYME G990 4X from Genencor International Inc.) or *Rhizopus* species (trade name CU.CONC. from Shin Nihon Chemicals, Japan and trade name GLUCZYME from Amano Pharmaceuticals, Japan).

A particular group of enzymes having glucoamylase activity are granular starch hydrolyzing enzyme(s) GSHE (See, Tosi et al., (1993) *Can. J. Microbiol.* 39:846 –855). GSHEs not only have glucoamylase activity, but also are able to hydrolyze granular (raw) starch. GSHEs have been recovered from fungal cells such as *Humicola* sp., *Aspergillus* sp. and *Rhizopus* sp. A *Rhizopus oryzae* GSHE has been described in Ashikari et al., (1986) *Agric. Biol. Chem.* 50:957-964 and USP 4,863,864. A *Humicola grisea* GSHE has been described in Allison et al., (1992) *Curr. Genet.* 21:225-229 and European Patent No. 171218. The gene encoding this enzyme is also known in the art as *gla1*. An *Aspergillus awamori* var. *kawachi* GSHE has been described by Hayashida et al., (1989) *Agric. Biol. Chem.* 53:923-929. An *Aspergillus shirousami* GSHE has been described by Shibuya et al., (1990) *Agric. Biol. Chem.* 54:1905-1914.

In one embodiment a GSHE may be derived from a strain of *Humicola grisea*, particularly a strain of *Humicola grisea* var. *thermoidea* (see, USP 4,618,579).

In some preferred embodiments, the GSHE is recovered from fungi including ATCC 16453, NRRL 15219, NRRL 15220, NRRL 15221, NRRL 15222, NRRL 15223, NRRL 15224 and NRRL 15225 as well as genetically altered strains thereof. (EP 0 171218).

In one embodiment, a GSHE may be derived from a strain of *Aspergillus awamori*, particularly a strain of *A. awamori* var. *kawachi* (See, Hayashida et al., (1989) *Agric. Biol. Chem.* 53:923-929).

In another embodiment, a GSHE may exhibit a maximum pH activity within a pH range of 4 to 7.5 and within a pH range of 5.0 to 7.5 and a maximum activity in the temperature range of 50 to 60°C.

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In one embodiment, the GSHE has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 93%, 95%, 97%, 98% and 99% sequence identity with the amino acid sequence set forth in SEQ ID NO: 3. In another embodiment, the GSHE comprises an amino acid sequence having at least 80% sequence identity with the sequence set forth in SEQ ID NO: 3. In other embodiments, the GSHE comprising the amino acid sequence of SEQ ID NO: 3 or a GSHE having at least 80% sequence identity with the sequence of SEQ ID NO: 3 is encoded by a polynucleotide having at least 70%, 80%, 85%, 90%, 93%, 95%, 97%, 98% and 99% sequence identity with SEQ ID NO: 1.

In another embodiment, the GSHE has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 93%, 95%, 97%, 98% and 99% sequence identity with the amino acid sequence set forth in SEQ ID NO: 6. In another embodiment, the GSHE comprises an amino acid sequence having at least 80% sequence identity with the sequence set forth in SEQ ID NO: 6. In other embodiments, the GSHE comprising the amino acid sequence of SEQ ID NO: 6 or a GSHE having at least 80% sequence identity with the sequence of SEQ ID NO: 6 is encoded by a polynucleotide having at least 70%, 80%, 85%, 90%, 93%, 95%, 97%, 98% and 99% sequence identity with SEQ ID NO: 4.

A polynucleotide or polypeptide having a certain percent (e.g., 80%, 85%, 90% or 99%) of sequence identity with another sequence means that when aligned, that percent of bases or amino acid residues are the same in comparing the two sequences. This alignment and the percent homology or identity can be determined using any suitable software program known in the art, for example those described in Current Protocols in Molecular Biology (Ausubel et al., eds 1987 Supplement 30, section 7.7.18). Preferred programs include GCG Pileup program, FASTA and BLAST. Another preferred alignment program is ALIGN Plus and TFASTA.

One skilled in the art will recognize that sequences encompassed by the invention are also defined by the ability to hybridize under stringent hybridization conditions with the exemplified GSHE sequences (e.g. SEQ ID NO: 1 or SEQ ID NO: 4). A nucleic acid is hybridizable to another nucleic acid sequence when a single stranded form of the nucleic acid can anneal to the other nucleic acid under appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known in the art (See, e.g. Sambrook (1989) *supra*, particularly chapters 9 and 11). In some embodiments, stringent conditions correspond to a T<sub>m</sub> of 65°C and 0.1x SSC, 0.1% SDS. In a further embodiment, a GSHE enzyme may be derived from a strain of *Rhizopus*. Such as the enzyme derived from the Koji strain of *R. niveus* (sold under the trade name "CU CONC") or the enzyme from *Rhizopus* sold under the trade name GLUCZYME.

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In a preferred embodiment, the GHSE used in a method or composition encompassed by the invention is a recombinantly expressed GSHE obtained from a filamentous fungal strain, which has been genetically engineered to express a heterologous polynucleotide that encodes a GSHE derived from a source other than the host strain.

In some embodiments the filamentous fungal strain is a strain of *Aspergillus sp.*, *Trichoderma sp.*, *Fusarium sp.*, or *Penicillium sp.* Particularly preferred fungal hosts include *A. nidulans*, *A. awamori*, *A. oryzae*, *A. aculeatus*, *A. niger*, *A. japonicus*, *T. reesei*, *T. viride*, *F. oxysporum*, and *F. solani*. *Aspergillus* strains are disclosed in Ward *et al.*, (1993) *Appl. Microbiol. Biotechnol.* 39:738-743 and Goedegebuur *et al.*, (2002) *Curr. Gene* 41:89-98. In a most preferred embodiment, the host is a *Trichoderma* strain and particularly a *T. reesei* strain. Strains of *T. reesei* are known and nonlimiting examples include ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56765, ATCC NO. 56767 and NRRL 15709. In some preferred embodiments, the host strain is a derivative of RL-P37. RL-P37 is disclosed in Sheir-Neiss *et al.*, (1984) *Appl. Microbiol. Biotechnol.* 20:46 – 53.

A host strain which expresses rGSHE may have been previously manipulated through genetic engineering. In some embodiments, various genes of the fungal host have been inactivated. These genes include, for example genes encoding cellulolytic enzymes, such as endoglucanases (EG) and exocellobiohydrolases (CBH) (e.g., *cbh1*, *cbh2*, *egl1*, *egl2* and *egl3*). US Patent No. 5,650,322 discloses derivative strains of RL-P37 having deletions in the *cbh1* gene and the *cbh2* gene.

In some embodiments, the fungal host has been genetically engineered to comprise a polynucleotide encoding a GSHE derived from *Humicola grisea*. In one embodiment the rGSHE will have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 93%, 95%, 97%, 98% and 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. In other embodiments, a polynucleotide encoding the GSHE of SEQ ID NO: 3 will have at least 70%, 80%, 85%, 90%, 95%, 97% and 98% sequence identity with the sequence of SEQ ID NO: 1. In a particularly preferred embodiment, the GSHE is expressed in a *Trichoderma reesei* strain and the produced protein has at least 80%, 85%, 90%, 95%, 97% and 98% sequence identity with the sequence of SEQ ID NO: 3.

In other embodiments, the fungal host has been genetically engineered to express a polynucleotide encoding a GSHE derived from *Aspergillus awamori*. In one embodiment, the rGSHE will have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 93%,



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95%, 97%, 98% and 99% sequence identity to the amino acid sequence set forth in SEQ ID NO. 6. In other embodiments, a polynucleotide encoding the GSHE of SEQ ID NO: 6 will have at least 70%, 80%, 85%, 90%, 95%, 97% and 98% sequence identity with the sequence of SEQ ID NO: 4. In a particularly preferred embodiment, the GSHE is  
5 expressed in a *Trichoderma reesei* strain and the produced protein has at least 80%, 85%, 90%, 95%, 97% and 98% sequence identity with the sequence of SEQ ID NO: 6.

In some embodiments, the level of glycosylation of the recombinantly expressed GSHE is different than the level of glycosylation of the corresponding native GSHE (e.g., GSHE which was originally derived from *H. grisea* or *A. awamori* has a different level of  
10 glycosylation than the level of glycosylation of the GSHE expressed in *Trichoderma*). In one embodiment, the level of glycosylation is different even if the rGSHE has at least 80%, 85%, 90%, 95% amino acid identity to the corresponding native GSHE. In some embodiments, a rGSHE expressed in *Trichoderma* and particularly a strain of *T. reesei* has a different level of glycosylation than the level from the corresponding nGSHE. In  
15 other embodiments, the level of glycosylation is higher, while in other embodiments it is lower.

For example, the level of glycosylation for rGSHE may be at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, or 65% less than the level of glycosylation of the corresponding nGSHE. In other embodiments, the level of  
20 glycosylation of an expressed rGSHE may be at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 100%, 125%, 150%, 175%, 200%, 225% or 250% greater than the level of a corresponding nGSHE.

In another embodiment, the recombinantly produced GSHE produced according to the invention may have greater stability at lower pH levels than the corresponding native  
25 GSHE at optimum temperature levels. More specifically, a rGSHE expressed in *Trichoderma*, which was originally derived from *Humicola grisea* var. *thermoidea*, has a greater stability at pH levels of 3.5 to 4.0 compared to a corresponding nGSHE at a temperature of 45 – 55°C. Under some conditions the stability of rGSHE and particularly *H. grisea* var. *thermoidea* SEQ ID NO: 1 expressed in *Trichoderma reesei* is more than  
30 double the level of stability of nGSHE.

#### Vectors and Fungal Transformation:

According to the invention, a DNA construct comprising a polynucleotide encoding a GSHE encompassed by the invention is constructed to transfer GSHE into a host cell.  
35 Thus, a GSHE polynucleotide which can be expressed in enzyme form may be introduced

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into a host cell using a vector, particularly an expression vector which comprises a regulatory sequence operably linked to a GSHE coding sequence.

The vector may be any vector which when introduced into a fungal host cell is integrated into the host cell genome and is replicated. Reference is made to the Fungal Genetics Stock Center Catalogue of Strains ([www.FGSC.net](http://www.FGSC.net)) for a list of vectors. Also, examples of suitable expression vectors may be found in Sambrook et al., (1989) *supra*, and Ausubel (1987) *supra*, and more specifically reference is made to van den Hondel et al. (1991) in Bennett and Lasure Eds. MORE GENE MANIPULATIONS IN FUNGI, Academic Press pp. 396-428. Particularly useful vectors include pFB6, pBR322, PUC18, pUC100 and pENTR/D.

In some embodiments, a nucleic acid encoding a GSHE is operably linked to a suitable promoter, which shows transcriptional activity in the fungal host cell. The promoter may be derived from genes encoding proteins either homologous or heterologous to the host cell. Preferably, the promoter is useful in a *Trichoderma* host and suitable nonlimiting examples of promoters include *cbh1*, *cbh2*, *egl1*, *egl2*. In one embodiment, the promoter is one that is native to the host cell. For example, when *T. reesei* is the host, the promoter would be a native *T. reesei* promoter. In a preferred embodiment, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235. An inducible promoter is a promoter that is active under environmental or developmental regulation. In another embodiment the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from *A. awamori* and *A. niger* glucoamylase genes (See, Nunberg et al., (1984) *Mol. Cell Biol.* 4:2306-2315 and Boel et al., (1984) *EMBO J.* 3:1581-1585). Also, the promoters of the *T. reesei xln1* gene and the *cellobiohydrolase 1* gene may be useful (EPA 137280A1).

In some embodiments, the GSHE coding sequence is operably linked to a signal sequence. The DNA encoding the signal sequence is preferably that which is naturally associated with the GSHE gene to be expressed. Preferably, the signal sequence is encoded by a *Humicola grisea* or *Aspergillus awamori* gene which encodes a GSHE. More preferably the signal sequence has at least 90%, at least 95%, at least 97%, and at least 99% sequence identity to the signal sequence depicted in Figure 2A and 6A. In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into a fungal host cell are derived from the same source. For example, in some embodiments, the signal sequence is the *cdh1* signal sequence which is operably linked to a *cdh1* promoter.

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In some embodiments, the expression vector also includes a termination sequence. In one embodiment, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is homologous to the host cell. A particularly suitable terminator sequence is *cbh1* derived from a *Trichoderma* strain and particularly *T. reesei*. Other useful fungal terminators include the terminator from *A. niger* or *A. awamori* glucoamylase gene (Nunberg *et al.* (1984) *supra*, and Boel *et al.*, (1984) *supra*).

In some embodiments, an expression vector includes a selectable marker. Examples of preferred selectable markers include ones which confer antimicrobial resistance (e.g., hygromycin and phleomycin). Nutritional selective markers also find use in the present invention including those markers known in the art as *amdS*, *argB* and *pyr4*. Markers useful in vector systems for transformation of *Trichoderma* are described in Finkelstein, chapter 6 in BIOTECHNOLOGY OF FILAMENTOUS FUNGI, Finkelstein *et al.* Eds. Butterworth-Heinemann, Boston, MA (1992), Chap. 6. and Kinghorn *et al.* (1992) APPLIED MOLECULAR GENETICS OF FILAMENTOUS FUNGI, Blackie Academic and Professional, Chapman and Hall, London. In a preferred embodiment, the selective marker is the *amdS* gene, which encodes the enzyme acetamidase allowing transformed cells to grow on acetamide as a nitrogen source. (See, Kelley *et al.*, (1985) *EMBO J.* 4:475-479 and Penttilä *et al.*, (1987) *Gene* 61:155 – 164.

An expression vector comprising a polynucleotide encoding a GSHE may be any vector which is capable of replicating autonomously in a given fungal host organism or of integrating into the DNA of the host. In some embodiments, an expression vector is a plasmid. In preferred embodiments, two types of expression vectors for obtaining expression of genes are contemplated.

The first expression vector comprises DNA sequences in which the promoter, GSHE coding region, and terminator all originate from the gene to be expressed. In some embodiments, gene truncation is obtained by deleting undesired DNA sequences (e.g., coding for unwanted domains) to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences.

The second type of expression vector is preassembled and contains sequences required for high-level transcription and a selectable marker. In some embodiments, the coding region for a GSHE gene or part thereof is inserted into this general-purpose expression vector such that it is under the transcriptional control of the expression constructs promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh1* promoter.

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Methods used to ligate a vector comprising a polynucleotide encoding a GSHE, a promoter, a terminator and other sequences and to insert them into a suitable vector are well known in the art. Linking is generally accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide linkers are used in accordance with conventional practice. (See, Sambrook (1989) *supra*, and Bennett and Lasure, MORE GENE MANIPULATIONS IN FUNGI, Academic Press, San Diego (1991) pp 70 – 76.) Additionally, vector can be constructed using known recombination techniques (e.g. Invitrogen Life Technologies, Gateway Technology).

Where it is desired to obtain a fungal host cell having one or more inactivated genes known methods may be used (See, US Patent No. 5,246,853; US Patent No. 5,475,101 and WO 92/06209). Gene inactivation may be accomplished by complete or partial deletion, by insertional inactivation or by any other means which renders a gene nonfunctional for its intended purpose (such that the gene is prevented from expression of a functional protein). Any gene from a *Trichoderma* sp. or other filamentous fungal host, which has been cloned can be deleted, for example *cbh1*, *cbh2*, *egl1* and *egl2*. In some embodiments, gene deletion is accomplished by inserting a form of the desired gene to be inactivated into a plasmid by known methods. The deletion plasmid is then cut at an appropriate restriction enzyme site(s), internal to the desired gene coding region and the gene coding sequence or part thereof is replaced with a selectable marker, Flanking DNA sequences from the locus of the gene to be deleted remain on either side of the marker (preferably about between 0.5 to 2.0 kb). An appropriate deletion plasmid will generally have unique restriction enzyme sites present therein to enable the fragment containing the deleted gene, including the flanking DNA sequences and the selectable marker gene to be removed as a single linear piece.

Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, including lipofection mediated and DEAE-Dextrin mediated transfection; incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are taught in Ausubel et al., (1987), *supra* chapter 9 and Sambrook (1989) *supra*. More specifically methods of transformation for filamentous fungi are disclosed in Campbell et al., (1989) *Curr. Genet.* 16:53-56. Specifically, to effect the expression of heterologous protein in *Trichoderma* reference is made to USP 6,022,725; USP 6,268,328; Harkki et al. (1991); *Enzyme Microb. Technol.* 13:227-233; Harkki et al., (1989) *Bio Technol.* 7:596-603; EP 244,234; and EP 215,594. Reference is also made to Nevalainen et al., "The Molecular

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*Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*", in MOLECULAR INDUSTRIAL MYCOLOGY, Eds. Leong and Berka, Marcel Dekker Inc., NY (1992) pp. 129 - 148. Reference is also made to Cao et al., (2000) *Sci.* 9:991 - 1001 for transformation of *Aspergillus* strains.

5 Preferably genetically stable transformants may be constructed with vector systems whereby the nucleic acid encoding GSHE is stably integrated into a host strain chromosome. Transformants may then be purified by known techniques.

In one nonlimiting example, stable transformants including an *amdS* marker are distinguished from unstable transformants by their faster growth rate and the formation of  
10 circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on solid non-selective medium (*i.e.* lacking acetamide), harvesting spores from this culture medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium containing  
15 acetamide. Alternatively, other methods known in the art may be used to select transformants.

In one specific embodiment, the preparation of *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelia. (See, Campbell et al., (1989) *Curr. Genet.* 16:53-56). In some embodiments, the mycelia are obtained from germinated  
20 vegetative spores and treated with an enzyme that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M. It is preferable to use about a 1.2 M solution of sorbitol in the  
25 suspension medium.

Uptake of DNA into the host *Trichoderma sp.* strain is dependent upon the calcium ion concentration. Generally between about 10 mM  $\text{CaCl}_2$  and 50 mM  $\text{CaCl}_2$  is used in an uptake solution. Besides the need for the calcium ion in the uptake solution, other items generally included are a buffering system such as TE buffer (10 Mm Tris, pH 7.4; 1 mM  
30 EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG). It is believed that the polyethylene glycol acts to fuse the cell membranes thus permitting the contents of the medium to be delivered into the cytoplasm of the *Trichoderma sp.* strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA tenderly integrated into the host  
35 chromosome.

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Usually a suspension containing the *Trichoderma* sp. protoplasts or cells that have been subjected to a permeability treatment at a density of  $10^5$  to  $10^7$ /mL, preferably  $2 \times 10^6$ /mL are used in transformation. A volume of 100  $\mu$ L of these protoplasts or cells in an appropriate solution (e.g., 1.2 M sorbitol; 50 mM  $\text{CaCl}_2$ ) are mixed with the desired DNA.

5 Generally a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. However, it is preferable to add about 0.25 volumes to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like may also be added to the uptake solution and aid in transformation. Similar procedures are available  
10 for other fungal host cells. See, for example, U.S. Patent Nos. 6,022,725 and 6,268,328, the contents of which are hereby incorporated by reference.

Generally, the mixture is then incubated at approximately  $0^\circ\text{C}$  for a period of between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 is  
15 generally added in volumes of 5 to 15 times the volume of the transformation mixture; however, greater and lesser volumes may be suitable. The 25% PEG 4000 is preferably about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture is then incubated either at room temperature or on ice before the addition of a sorbitol and  $\text{CaCl}_2$  solution. The protoplast suspension is then further added  
20 to molten aliquots of a growth medium. This growth medium permits the growth of transformants only.

#### Cell culture -

Appropriate host cells are generally cultured in a standard medium containing  
25 physiological salts and nutrients, such as described in Pourquie, J. et al., BIOCHEMISTRY AND GENETICS OF CELLULOSE DEGRADATION, eds. Aubert, J. P. et al., Academic Press, pp. 71-86, 1988 and Ilmen, M. et al., (1997) *Appl. Environ. Microbiol.* 63:1298-1306. Also reference is made to common commercially prepared media such as Yeast Malt Extract (YM) broth, Luria Bertani (LB) broth and Sabouraud Dextrose (SD) broth.

30 Culture conditions are also standard, e.g., cultures are incubated at approximately  $28^\circ\text{C}$  in appropriate media in shaker cultures or fermenters until desired levels of GSHE expression are achieved. Preferred culture conditions for a given filamentous fungus may be found in the scientific literature and/or from the source of the fungi such as the American Type Culture Collection and Fungal Genetics Stock Center ([www.FGSC.net](http://www.FGSC.net)).

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After fungal growth has been established, the cells are exposed to conditions effective to cause or permit the expression of a GSHE and particularly a GSHE as defined herein. In cases where a GSHE coding sequence is under the control of an inducible promoter, the inducing agent, e.g., a sugar, metal salt or antibiotics, is added to the medium at a concentration effective to induce GSHE expression.

#### Industrial Uses of the rGSHE – Fermentations -

In some embodiments of the present invention, fungal cells expressing a heterologous GSHE are grown under batch or continuous fermentation conditions. A classical batch fermentation is a closed system, wherein the composition of the medium is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO<sub>2</sub>. Batch and fed-batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

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Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

#### Identification of GSHE Activity -

In order to evaluate the expression of a GSHE by a cell line that has been transformed with a heterologous polynucleotide encoding a GSHE encompassed by the invention, assays can be carried out at the protein level, the RNA level or by use of functional bioassays particular to glucoamylase activity and/or production. In general, assays employed to analyze the expression of a GSHE include, Northern blotting, dot blotting (DNA or RNA analysis), RT-PCR (reverse transcriptase polymerase chain reaction), or *in-situ* hybridization, using an appropriately labeled probe (based on the nucleic acid coding sequence) and conventional Southern blotting and autoradiography. In addition, the production and/or expression of a GSHE may be measured in a sample directly, for example, by assays directly measuring reducing sugars such as glucose in the culture media and by assays for measuring glucoamylase activity, expression and/or production. Substrates useful for assaying GSHE activity include granular starch substrates. For example, glucose concentration may be determined by any convenient method such as by using glucose reagent kit No 15-UV (Sigma Chemical Co.) or an instrument such as Technicon Autoanalyzer. Also reference is made to glucose oxidase kits and glucose hexose kits commercially available from Instrumentation Lab. (Lexington, MA). Glucoamylase activity may be assayed by the 3,5-dinitrosalicylic acid (DNS) method (See, Goto et al., (1994) Biosci. Biotechnol. Biochem. 58:49 - 54). In one nonlimiting example, a rGSHE has the ability to hydrolyze granular starch in a 15% starch solids suspension in water to a solution of saccharides of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% wt glucose, dry substance basis.



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In an embodiment of the invention, the GSHE expressed by a recombinant host will be greater than 1 gram protein per liter (g/L) of culture media. Preferably in some embodiments, the host is a *Trichoderma* or an *Aspergillus* host. In some embodiments, the amount of GSHE expressed by a recombinant *Trichoderma* host will be greater than 2 g/L of culture media. In other embodiments, the amount of GSHE expressed by a recombinant *Trichoderma* host will be greater than 5 g/L of culture media. Yet in other embodiments the amount of GSHE expressed by a recombinant *Trichoderma* host will be greater than 10 g/L of culture media. The amount of expressed GSHE may in some instances be greater than 20 g/L, greater than 25 g/L, greater than 30g/L and greater than 50 g/L of culture media.

In addition, protein expression, may be evaluated by immunological methods, such as immunohistochemical staining of cells, tissue sections or immunoassay of tissue culture medium, e.g., by Western blot or ELISA. Such immunoassays can be used to qualitatively and quantitatively evaluate expression of a GSHE. The details of such methods are known to those of skill in the art and many reagents for practicing such methods are commercially available.

Exemplary assays include ELISA, competitive immunoassays, radioimmunoassays, Western blot, indirect immunofluorescent assays and the like. In general, commercially available antibodies and/or kits may be used for the quantitative immunoassay of the expression level of a GSHE.

#### Methods for Purifying GSHE -

In general, a GSHE (nGSHE or rGSHE) produced in cell culture is secreted into the medium and may be purified or isolated, e.g., by removing unwanted components from the cell culture medium. In some cases, a GSHE may be produced in a cellular form necessitating recovery from a cell lysate. In such cases the enzyme is purified from the cells in which it was produced using techniques routinely employed by those of skill in the art. Examples include, but are not limited to, affinity chromatography (Tilbeurgh *et al.*, (1984) *FEBS Lett.* 16:215); ion-exchange chromatographic methods (Goyal *et al.*, (1991) *Biores. Technol.* 36:37; Fliess *et al.*, (1983) *Eur. J. Appl. Microbiol. Biotechnol.* 17:314; Bhikhabhai *et al.*, (1984) *J. Appl. Biochem.* 6:336; and Ellouz *et al.*, (1987) *Chromatography* 396:307), including ion-exchange using materials with high resolution power (Medve *et al.*, (1998) *J. Chromatography A* 808:153; hydrophobic interaction chromatography (Tomaz and Queiroz, (1999) *J. Chromatography A* 865:123; two-phase partitioning (Brumbauer, *et al.*, (1999) *Bioseparation* 7:287); ethanol precipitation; reverse

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phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, e.g., Sephadex G-75. The degree of purification desired will vary depending on the use of the GSHE. In some embodiments, purification will not be necessary..

5 In some embodiments, the recombinantly expressed GSHE is from a *Trichoderma* host. In other embodiments the *Trichoderma* host expresses a heterologous polynucleotide, which encodes a GSHE from a *Humicola grisea* strain, particularly a strain of *Humicola grisea* var. *thermoidea*. In other embodiments, the *Trichoderma* expresses a recombinant GSHE wherein the heterologous polynucleotide encodes a GSHE having at  
10 least 50% sequence identity with the sequence of SEQ ID NO:3.

In some embodiments, *Trichoderma* host expresses a heterologous polynucleotide, which encodes a GSHE from a *Aspergillus awamori* strain, particularly a strain of *A. awamori* var. *kawachi*. In other embodiments, the *Trichoderma* expresses a recombinant GSHE wherein the heterologous polynucleotide encodes a GSHE having at  
15 least 50% sequence identity with the sequence of SEQ ID NO:6.

#### Composition and Process Conditions -

Whether the GSHE is supplied in a cell free extract or supplied in the culture medium (fermentation broth), which includes fungal cells that express and secrete GSHE,  
20 the granular starch substrate, preferably in slurry form is contacted with the GSHE and alpha amylase essentially simultaneously (referred to herein as simultaneously) to hydrolyze the granular starch and produce a glucose syrup. The hydrolysis of the granular starch is a one-step process.

A GSHE may be added to a composition comprising an alpha amylase and a  
25 granular starch substrate in an amount of between about 0.01 to 10.0 GSHE U/g starch dry solids of a slurry adjusted to 10 - 55% dry solids. In some embodiments, the GSHE is added in an amount of between about 0.01 and 5.0 GSHE U/g; about 0.01 and 2.0 GSHE U/g; about 0.01 and 1.5 GSHE U/g; about 0.05 and 1.5 GSHE U/g; about 0.1 and 5.0 GSHE U/g; about 0.1 and 1.0 GSHE U/g; about 0.25 and 2.5 GSHE U/g; about 0.5 and  
30 5.0 GSHE U/g; and about 0.5 and 1.0 GSHE U/g of such solution. Also in some preferred embodiments, the GSHE is added in an amount of between about 0.05 and 1.5 GSHE U/g of such solution, also between 0.1 and 2.0 GSHE U/g and also between about 0.1 and 1.0 GSHE U/g.

In further embodiments, a GSHE is added to a granular starch slurry composition  
35 essentially simultaneously with alpha amylase wherein the slurry is adjusted to 10 to about

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55% ds, preferably 20 – 45%ds and also 25 – 45% ds. In certain embodiments, the alpha amylase comprising the composition is added in a range of about 0.01 to 1.0 kg of GZYME 997 per metric ton of starch.

In one embodiment, the granular starch substrate is contacted with a GSHE  
5 wherein the GSHE is available as a cell free filtrate (such that the GSHE is isolated from the culture medium). In another embodiment, the granular starch substrate is contacted with a GSHE, wherein the GSHE is available in a culture medium containing the secreted GSHE and fungal cells. Preferably, the GSHE will be secreted from a *Trichoderma reesei* containing a heterologous polynucleotide encoding a polypeptide having granular starch  
10 hydrolyzing activity and at least 90%, at least 95% and at least 98% sequence identity with the sequence of SEQ ID NO: 3 or SEQ ID NO: 6.

The methods of the invention are conducted at a temperature equal to or below the gelatinization temperature of the granular starch of the substrate. In some embodiments, the method is conducted at a temperature of at least about 25°C, 30°C, 35°C, 40°C, 45°C,  
15 50°C, 55°C, 60°C, 65°C, 70°C and 75°C. In other embodiments, the method is conducted at a temperature less than about 65°C and also less than about 60°C. In other embodiments, the method is conducted at a temperature of between about 30°C and 65°C; also between about 35°C and 65°C; between about 40°C and 65°C; between about 45°C and 65°C; and between about 50°C and 65°C. The exact temperature used in  
20 accordance with the method depends upon the specific starch substrate and further may depend upon the particular plant variety. In some embodiments, when corn is the granular starch substrate the temperature is conducted at about between 55°C and 65° and more particularly between 60°C and 65°C.

Table 2 illustrates the general starch gelatinization temperature ranges for a  
25 number of starches. The table has been compiled from various sources and is not meant to limit the invention, but is provided as a guide.

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TABLE 2

Temperature Range for the Gelatinization of Starches

Starch	Gelatinization Temperature Range °C
Barley	52 – 59
Wheat	58 – 64
Rye	57 – 70
Corn (maize)	62 – 72
High amylose corn	67 – 80
Rice	68 – 77
Sorghum	68 – 77
Potato	58 – 68
Tapioca	59 – 69
Sweet Potato	58 – 72

( J.J.M. Swinkels pg 32 - 38 in STARCH CONVERSION TECHNOLOGY, Eds Van Beynum et al., (1985) Marcel Dekker Inc. New York and The Alcohol Textbook 3<sup>rd</sup> ED. A reference for the beverage, fuel and industrial alcohol industries, Eds Jacques et al., (1999) Nottingham University Press, UK )

The pH range at which the methods of the invention is conducted is in the range of about pH 3.0 to pH 6.5; also the range of pH 3.5 to pH 6.5; the range of pH 4.0 to pH 6.5; and the range of pH 4.5 to pH 6.0 are used in the methods. The pH range is somewhat dependent of the specific enzymes and one skilled in the art would be able to determine the best pH range for conducting the methods without undue experimentation. In some embodiments, when corn is the substrate the pH range is about pH 4.5 to pH 6.0 and also about pH 5.0 to pH 5.5.

In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 94%, 95%, 96%, 97%, 98% and 99% of the dry solids of the granular starch is converted into a composition of glucose syrup. In some embodiments, the granular starch substrate is completely hydrolyzed. In certain embodiments, at least 90% of the granular starch substrate is hydrolyzed in a time period of 24 hours. In other embodiments, at least 95% of the granular starch substrate is hydrolyzed in a time period of 24 hours. In other embodiments, the dextrose syrup produced according to the invention will be about 32 to 46% ds syrup containing at least 90% glucose.

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In some embodiments, the period of time required to hydrolyze the granular starch to produce a glucose syrup is from about 2 to 100 hours. In some embodiments, the period of time is about 5 to 100 hours. In other embodiments, the period of time is from about 10 to 100 hours. In still other embodiments, the period of time is from 5 to 50 hours. In other embodiments, the period of time is at least about 10 hours but less than about 50 hours. In preferred embodiments, the one-step process will be conducted from 2 to 100 hours and in some embodiments, the process will be conducted from 5 hours to 50 hours.

Preferably, the yield of glucose in the solublized composition (glucose percent of the total solublized dry solids) is at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99% and 99.5%. More preferably, the yield is at least about 95% and most preferably, the yield is at least about 96%.

The exact amounts of the components encompassed by the composition and methods of the invention depend on the combination of enzymes applied as well as the type of granular starch processed. In some embodiments, the ratio of alpha amylase units to GSHE units (alpha amylase:GSHE) will be in the range of 15:1 to 1:15 and in some embodiments in the range of 10:1 to 1:10. In other embodiments, the ratio will be in the range of 5:1 to 1:5 and in further embodiments, the alpha amylase:GSHE will be 4:1 to 1:4. In preferred embodiments, the ratio will be about 2:1 to 1:4 and most preferably about 2:1 to 1:2.

The one-step process encompassed by the invention can include the addition of further ingredients without reducing the effectiveness of the hydrolysis of granular starch. These further ingredients include but are not limited to other enzymes, such as cellulases, proteases, pullulanases, hemicellulase, xylanases and the like.

The glucose produced according to the method of the invention may be separated from the reaction mixture by methods known in the art. Some of these methods include centrifugation, conventional filtration methods and preferably membrane separation processes. Also mentioned is the use of ultrafiltration membrane systems. In one preferred embodiment, the glucose syrup is separated by filtration using a molecular weight cut-off (MWCO) ultrafiltration membrane. These membranes are known in the art. In some embodiments, the membrane could be 1,000 to 1,000,000 MWCO. In other embodiments, the separation membrane may be a 0.1 to 1.0 microfilter type membrane.

Further conversion of glucose to desired end products -

In a method encompassed by the invention, glucose syrup is the preferred end product. However, the glucose may be further purified to yield crystalline dextrose by known methods.

5       The glucose may also be converted to other desired end products. Conversion of glucose to other desired end products may be accomplished by any suitable method such as, enzymatic or chemical methods. In one embodiment, conversion is accomplished by bioconversion of glucose by contacting glucose obtained according to the invention with a microorganism capable of converting the glucose to an end product. The contacting step  
10       may be a sequential step, wherein the glucose syrup produced by the method of the invention is then contacted with a microorganism to produce an end product, or the contacting step may be a simultaneous step, wherein the granular starch substrate is contacted with the GSHE and alpha amylase enzyme in combination with a microorganism capable of converting the glucose syrup produced by the enzyme  
15       conversion to an end-product. The microorganism may be a wild-type, mutated or recombinant microorganism. In some embodiments, the desired end products are fructose, ascorbic acid (ASA) intermediates, such as gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, 2-keto-L-gulonic acid, idonic acid, erythorbic acid and ascorbic acid; ethanol, 1,3-propanediol, monosodium glutamate, amino acids, sugars alcohols, organic  
20       acids, and indigo.

When fructose is the desired end-product, the glucose syrup obtained according to the present invention may be enzymatically converted to a fructose syrup by a glucose isomerase. In some embodiments, the glucose isomerase is immobilized. Contemplated glucose isomerases include those commercially available such as G ZYME™ G993 liquid  
25       and GENSWEET™ (Genencor International, Inc. ) and SWEETZYME T (Novozyme). (See, e.g. US Patent No. 3,939,041 and US Patent No. 4,687,742).

When ASA intermediates and ASA are the desired end-products, the glucose syrup obtained according to the present invention may be enzymatically bioconverted to gluconate using for example, glucose dehydrogenase (or glucose oxidase-catalase  
30       enzymes. Gluconate may be oxidized to 2,5-diketo-D- gluconate (DKG) by a DKG reductase. DKG may be reduced to 2-keto-L-gulonic acid (KLG) by a KLG reductase. KLG may then be converted to ASA. Methods for converting glucose to ASA and ASA intermediates are well known (See, for example US Patent No. 4,945,052, US Patent No. 5,008,193; US Patent No. 5,817,490 and US Patent No. 6,358,715).

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When 1, 3-propanediol is the desired end-product, glucose obtained according to the invention may be contacted with *E. coli* or other recombinant microorganisms (See, for example US Patent No. 6,013,494, U.S. Pat. No. 5,356,812).

When ethanol is the desired end-product, glucose may be contacted either  
5 sequentially or simultaneously with an ethanolgenic microorganism, such as the yeast *Saccharomyces cerevisiae* to obtain ethanol. (See, for example US Patent No. 4,316,956). Further examples of ethanolgenic microorganisms, which can be used in the methods of the invention, are those expressing alcohol dehydrogenase and pyruvate decarboxylase such as *Zymomonas mobilis* (See, for example US Patent Nos. 5,028,539; 5,424,202;  
10 5,487,989 and 5,514,583). Upon completion of the fermentation with yeast, the ethanol may be recovered, for example by distillation, and used for potable, fuel and industrial ethanol products. By-products of the fermentation include both liquid and solid material that can be separated and further used. For example, the recovered solid material, such as distiller's dried grain (DDG) and the mixture of DDS with liquid by-products to form  
15 distiller's dried grain with solubles (DDGS) may be used as an animal feed. The use of yeast for the production of ethanol during fermentation and ethanol production is further discussed in THE ALCOHOL TEXTBOOK, A REFERENCE FOR THE BEVERAGE, FUEL AND INDUSTRIAL ALCOHOL INDUSTRIES, 3<sup>rd</sup> Edition, Eds K.A. Jacques et al., 1999, Nottingham University Press, UK.

20 In some embodiments of the invention, when the glucose syrup is separated from the reaction mixture by for example, centrifugation or filtration as mentioned above, the remaining composition will include residual starch. The residual starch by-product may be used in various applications. For example, residual starch may be recycled and used as a component in a method according to the invention; the residual starch may be used as a  
25 carbon feedstock in further fermentations; the residual starch may be used in a conventional starch hydrolysis process; and the residual starch may be used as an ingredient for food formulations. One preferred embodiment of the invention comprises simultaneously contacting a granular starch substrate with a GSHE and an alpha amylase at a temperature below the gelatinization of the granular starch to hydrolyze the granular  
30 starch to obtain a glucose syrup, separating the glucose syrup from the reaction mixture to obtain a glucose syrup component and a by-product component which includes residual starch.

In some embodiments, according to the invention, when the residual starch is recycled in a recycling step and used in the method encompassed by the invention, the  
35 residual starch will be simultaneously contacted with a composition comprising a GSHE

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and an alpha amylase at a temperature below the gelatinization temperature of the granular starch substrate. The residual starch component may include enzymes that have been retained by the separation membrane and/or GSHE and alpha amylase enzymes that are newly added to the reactor. In some embodiments, the recycling step in  
5 combination with the simultaneous contacting step may be repeated numerous times and further may take place under continuous recycling conditions wherein the glucose syrup is separated by means known in the art. The contacting time of the various components in a reactor or vessel would be the same as outlined above that is from 2 to 100 hours. In some preferred embodiments, the residence time would be between 5 and 50 hours.

10 In the recycling step embodiment, the residual starch may be recycled to obtain glucose syrup. In one non-limiting example, a granular starch slurry (i.e. a corn starch slurry having 38 – 42% ds) may be hydrolyzed with a *Humicola* GSHE (i.e., 1.0 GSHE U/g) and SPEZYME ethyl (i.e., 0.6 AU/g) at a temperature of about 58 - 62°C and a pH of 5.0 to 6.0 for 20 – 24 hours, wherein at least 55% of the corn starch is hydrolyzed to  
15 produce a glucose syrup having at least 90% glucose. The residual starch may be recovered and resuspended and combined with a second round of GSHE and alpha amylase. In the second round, approximately at least 90% of the starch is hydrolyzed yielding at least 90% glucose. The glucose syrup may then be evaporated by means known in the art, such as by vacuum and then used as a glucose feed.

20 In the recycling step embodiment, the residual starch may be recycled to obtain end products other than glucose. For example, when the end product is ethanol, the granular starch substrate is contacted either separately and sequentially or simultaneously with GSHE, alpha amylase and an ethanolgenic organism to both hydrolyze the granular starch and produce ethanol, the ethanol may be recovered by distillation and the  
25 remaining material which includes both solid and liquid material including residual starch may be recycled and used with the GSHE and alpha amylase in further steps such that the recycling takes place under continuous recycling conditions.

## 30 EXPERIMENTAL

In the disclosure and experimental section which follows, the following abbreviations apply: rH-GSHE (*Humicola grisea* var. *thermoidea* GSHE expressed in *Trichoderma reesei*); wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per  
35 minute); H<sub>2</sub>O (water); dH<sub>2</sub>O (deionized water); bp (base pair); kb (kilobase pair);



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kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms);  
µl (microliters); ml and mL (milliliters); mm (millimeters); nm (nanometers); µm  
(micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW  
(molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); PAGE  
5 (polyacrylamide gel electrophoresis); Di (deionized); phthalate buffer (sodium phthalate in  
water, 20 mM, pH 5.0); Cerestar (Cerestar, Inc., a Cargill Inc., Minneapolis, MN);  
AVICELL®(FMC Corporation); SDS (sodium dodecyl sulfate); Tris  
(tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume);  
Genencor (Genencor International, Inc., Palo Alto, CA); Shin Nihon (Shin Nihon, Japan).

10

#### General Methods:

Starch Substrates - Purified and/or refined cornstarch, wheat starch and tapioca starch  
were used in the examples provided below.

15

Oligosaccharides Analysis - The composition of the reaction products of oligosaccharides  
was measured by high pressure liquid chromatographic method (Beckman System Gold  
32 Karat Fullerton, California, USA) equipped with a HPLC column (Rezex 8 u8% H,  
Monosaccharides), maintained at 50 °C fitted with a refractive index (RI) detector (ERC-  
20 7515A, RI Detector from The Anspec Company, Inc.). Dilute sulfuric acid (0.01 N) was  
used as the mobile phase at a flow rate of 0.6 ml per minute. Twenty microliter of 4.0%  
solution was injected on to the column. The column separates based on the molecular  
weight of the saccharides. For example a designation of DP1 is a monosaccharide, such  
as glucose; a designation of DP2 is a disaccharide, such as maltose; a designation of DP3  
25 is a trisaccharide, such as maltotriose and the designation "DP4\*" is an oligosaccharide  
having a degree of polymerization (DP) of 4 or greater.

Relative solubilization of the solids - A conventional low temperature jet cooking process  
was used to solublize the starch (USP 3,912,590). The measured BRIX was taken as  
30 100% solubilization of the starch under the defined parameters of starch to water ratio. In  
a typical jet cooking process, suspending 150 grams of starch in 350 grams of water made  
a 30% starch slurry. The pH was then adjusted to pH 5.8 using 10% NaOH. Thermostable  
alpha amylase, SPEZYME FRED (Genencor International Inc.) was added at 0.4 Kg/MT,  
ds and heated in a jet cooker maintained at 105° C for 8 min. The gelatinized starch was  
35 further hydrolyzed at 95°C for 90 min. An aliquot of the hydrolysate was withdrawn and  
centrifuged. The clear supernatant was used to measure the BRIX (ABBE Refractometer,

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American Optical Corporation, Scientific Instrument Division, Buffalo, New York ). The BRIX for 100% solublized starch for different starch substrate at 30% ds is given in Table 3 and used to calculate the percent relative solubilization of starch under different treatment conditions. Alternatively, BRIX for 100% solubilization under different conditions was determined by incubating 5 ml of an aliquot with 10 micro liter of SPEZYME FRED (Genencor International Inc.) at 95°C for 5 min. The high temperature treated sample was kept at 85°C for 2 hours. The insoluble solids were separated by centrifugation and the BRIX of the clear supernatant was measured.

TABLE 3

BRIX For Enzyme jet cooked starch substrate at 30% slurry

Enzyme jet cooked Starch substrate, 30% ds	Measured BRIX
Cornstarch	28.2
Wheat starch	27.9
Tapioca starch	28.5

### EXAMPLES

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

#### EXAMPLE 1

Expression of *Humicola grisea* var. *thermoidea* GSHE gene in *Trichoderma reesei*.

##### A. Cloning of the *Humicola grisea* var. *thermoidea* GSHE gene

Genomic DNA (SEQ ID NO:1) was extracted from frozen *Scytalidium thermophilum* (ATCC 16453, anamorph, *H. grisea* var. *thermoidea*) mycelia. The frozen mycelia were ground with dry ice in a coffee grinder and the DNA was extracted by the EasyDNA protocol (Invitrogen). An extra chloroform/phenol/isoamyl alcohol extraction was added to the standard protocol. PCR primers were designed, based on the NCBI database

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accession #M89475 sequence. The forward primer contained a motif for directional cloning into the pENTR/D vector (Invitrogen).

The sequence of the RSH003f primer was

CAACATGCATACCTTCTCCAAGCTCCTC (SEQ ID NO. 7) and the sequence of the

5 RSH004r primer was TTAACGCCACGAATCATTCA CCGTC (SEQ ID NO. 8).

The PCR product was cloned into pENTR/D, according to the Invitrogen Gateway system protocol. The vector was then transformed into chemically competent Top10 *E.coli* (Invitrogen) with kanamycin selection. Plasmid DNA from several clones was restriction digested to confirm the correct size insert. The *gla1* insert was sequenced  
10 (Sequetech, Mountain View, CA) from several clones. Plasmid DNA from one clone, pENTR/D\_N13, was added to the LR clonase reaction (Invitrogen Gateway system) with pTrex3g/*amdS* destination vector DNA. Recombination, in the LR clonase reaction, replaced the *CmR* and *ccdB* genes of the destination vector with the *H. grisea gla1* from the pENTR/D vector. This recombination directionally inserted *gla1* between the *cbhl*  
15 promoter and terminator of the destination vector. Recombination site sequences of 48 and 50 bp remained upstream and downstream, respectively, of *gla1*. An aliquot of the LR clonase reaction was transformed into chemically competent Top10 *E.coli* and grown overnight with carbenicillin selection. Plasmid DNA, from several clones, was digested with appropriate restriction enzymes to confirm the correct insert size. Plasmid DNA from  
20 clone, pTrex3g\_N13 (see Figures 3 and 4) was digested with *Xba1* to release the expression cassette including the *cbhl* promoter:*gla1*:*cbhl* terminator:*amdS*. This 6.6 kb cassette was purified by agarose gel extraction using standard techniques and transformed into a strain of *T. reesei* derived from the publicly available strain QM6a, as further described below.

25 The cassette was sequenced by Sequetech, Mountain View, CA and the DNA for GSHE is illustrated in Figure 1 (SEQ ID NO:1) and the amino acid sequence illustrated in Figure 2 (SEQ ID NOs:2 and 3).

#### B. Transformation of *T. reesei* -

30 Approximately 2 cm<sup>2</sup> of a plate of sporulated mycelia (grown on a PDA plate for 5 days at 30°C) was inoculated into 50ml of YEG (5g/L yeast extract plus 20g/L glucose) broth in a 250 ml, 4-baffle shake flask and incubated at 37°C for 16-20 hours at 200 rpm. The mycelia were recovered by transferring the liquid volume into 50ml conical tubes and spinning at 2500 rpm for 10 minutes. The supernatant was decanted. The mycelial pellet  
35 was transferred into a 250 ml, 0.22 micron CA Corning filter bottle containing 40ml of

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filtered  $\beta$ -D-glucanase solution and incubated at 30°C, 200 rpm for 2 hrs to generate protoplasts for transformation.

Protoplasts were harvested by filtration through sterile miracloth into a 50ml conical tube. They were pelleted by spinning at 2000 rpm for 5 minutes and aspirated. The  
5 protoplast pellet was washed once with 50ml of 1.2 M sorbitol, spun down, aspirated, and washed with 25ml of sorbitol/ $\text{CaCl}_2$ . Protoplasts were counted and then pelleted at 2000 rpm for 5 min, the supernate was decanted, and the protoplast pellet was resuspended in an amount of sorbitol/ $\text{CaCl}_2$  sufficient to generate a protoplast concentration of  $1.25 \times 10^8$  protoplasts per ml, generating a protoplast solution.

10 Aliquots of up to 20  $\mu\text{g}$  of expression vector DNA (in a volume no greater than 20  $\mu\text{l}$ ) were placed into 15ml conical tubes and the tubes were put on ice. Then 200 $\mu\text{l}$  of the protoplast suspension was added along with 50 $\mu\text{l}$  PEG solution to each transformation aliquot. The tubes were mixed gently and incubated on ice for 20 min. PEG solution (2 ml)  
15 was added to the transformation aliquot tubes, and these were incubated at room temperature for 5 minutes. Sorbitol/ $\text{CaCl}_2$  (4 ml) solution was added to the tubes (generating a total volume of 6.2 ml). The transformation mixture was divided into 3 aliquots each containing about 2ml. An overlay mixture was created by adding each of these three aliquots to three tubes of melted top agar (kept molten by holding at 50°C) and this overlay mixture was poured onto a transformation plate. The transformation plates  
20 were then incubated at 30°C for four to seven days.

The transformation was performed with *amdS* selection. Acetamide/sorbitol plates and top agar were used for the transformation. Top agar was prepared by the same Sorbitol/acetamide agar recipe as the plates, except that low melting agarose was used in place of Noble agar. Transformants were purified by transfer of isolated colonies to fresh  
25 selective media containing acetamide (i.e., Sorbitol/acetamide agar, without sorbitol).

With reference to the examples the solutions were prepared as follows.

1) 40 ml  $\beta$ -D-glucanase solution was made up in 1.2M sorbitol and included 600mg  $\beta$ -D-glucanase (InterSpex Products Inc., San Mateo, CA) and 400mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

30 2) 200 ml PEG mix contained 50g PEG 4000 (BDH Laboratory Supplies Poole, England) and 1.47g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  made up in  $\text{dH}_2\text{O}$ .

3) Sorbitol/  $\text{CaCl}_2$  contained 1.2M sorbitol and 50mM  $\text{CaCl}_2$ .

4) Acetamide/sorbitol agar:

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Part 1 - 0.6g acetamide (Aldrich, 99% sublime.), 1.68g CsCl, 20g glucose, 20g  $\text{KH}_2\text{PO}_4$ , 0.6g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.6g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1 ml 1000 x salts (see below), adjusted to pH 5.5, brought to volume (300 mls) with  $\text{dH}_2\text{O}$ , filter sterilized.

Part II - 20g Noble agar and 218g sorbitol brought to volume (700mls) with  $\text{dH}_2\text{O}$  and autoclaved.

Part II was added to part I for a final volume of 1L.

5) 1000 x Salts - 5g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.6g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 1.4g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  were combined and the volume was brought to 1L with  $\text{dH}_2\text{O}$ . The solution was filter sterilized.

C. Fermentation of *T. reesei* transformed with the *H. grisea* var. *thermoidea* GSHE gene.

In general, the fermentation protocol as described in Foreman *et al.* (Foreman *et al.* (2003) *J. Biol. Chem* 278:31988-31997) was followed. More specifically, duplicate fermentations were run for each of the strains displayed in Figure 5. 0.8 L of Vogels minimal medium (Davis *et al.*, (1970) *Methods in Enzymology* 17A, pg 79 - 143 and Davis, Rowland, *NEUROSPORA, CONTRIBUTIONS OF A MODEL ORGANISM*, Oxford University Press, (2000)) containing 5% glucose was inoculated with 1.5 ml frozen spore suspension. After 48 hours, each culture was transferred to 6.2L of the same medium in a 14L Biolafitte fermenter. The fermenter was run at 25°C, 750 RPM and 8 standard liters per minute airflow. One hour after the initial glucose was exhausted, a 25% (w/w) lactose feed was started and fed in a carbon limiting fashion to prevent lactose accumulation. The concentrations of glucose and lactose were monitored using a glucose oxidase assay kit or a glucose hexokinase assay kit with beta-galactosidase added to cleave lactose, respectively (Instrumentation Laboratory Co., Lexington, MA). Samples were obtained at regular intervals to monitor the progress of the fermentation. Collected samples were spun in a 50ml centrifuge tube at 3/4 speed in an International Equipment Company (Needham Heights, MA) clinical centrifuge.

Sample supernatants were run of 4 - 12% BIS-TRIS SDS -PAGE gels, under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 5. Lanes 3, 4 and 5 illustrate a 68 kD rGSHE band at different time periods.

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D. Assay of GSHE Activity from Transformed *Trichoderma reesei* Clones -

Enzyme activity – GSHE activity was determined as milligrams (mg) of reducing sugars released (measured as glucose equivalent) per minute (min) during an incubation of 5 ml of 10% granular cornstarch in a 0.1 M acetate buffer, pH 4.5, 50°C with an aliquot of the enzyme preparation. One unit of GSHE is defined as 1.0 mg of reducing sugar released per min under the assay conditions.

Native GSHE (nGSHE) from *Humicola grisea* var. *thermoidea* and recombinant GSHE produced from *T. reesei* were purified by standard techniques using hydrophobic interaction chromatography using phenyl-sepharose (Amersham Biosciences, Piscataway, NJ) followed by ion exchange chromatography using SP-sepharose (Amersham Biosciences, Piscataway, NJ). The recombinant GSHE initially expressed by *T. reesei* clones included two protein peak fractions in about equal concentrations. These peaks were labeled rGSHE1 and rGSHE2. The two peaks differed in mass by 1500D and by 0.3 pH units as measured by matrix assisted laser desorption and ionization (MALDI-TOF) on a voyageur mass spectrometer (Applied Biosystems, Foster City, CA) and an isoelectric focusing gel (SERVA Electrophoresis, GmbH, Heidelberg, Germany) according to manufacturer directions. Both rGSHE1 and rGSHE2 have the same specific activity as measured by the raw starch hydrolyzing assay and protein measurements using a MicroBCA protein assay kit (Pierce, Rockford, IL) and the percent solution extinction coefficient ( $A_{280} 0.1\% = 1.963$ ). After a period of time, measured at approximately 72 hours after initial rGSHE expression, only one form of rGSHE is represented (rGSHE3). (See, Table 4).

TABLE 4

Source of GSHE	Specific Activity GSHE Units/mg	% total carbohydrate
Native GSHE	9.0	1.12
rGSHE1/rGSHE2	8.0/8.0	2.70
rGSHE3	8.0	0.57

The % carbohydrate (CHO) of the GSHEs was determined by acid hydrolysis using 4N trifluoroacetic acid at 100°C for 5 hrs and measurements were made of the released reducing sugars using parahydroxybenzoic acid hydrazide.

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When initially expressed, the glycosylation of rGSHE1 and rGSHE2 was 2.70% of the total carbohydrate. However, after 72 hours, the level of glycosylation of rGSHE3 found in the medium was 0.57% total CHO. The level of glycosylation of native GSHE was 1.12%.

5

E. Comparison of native GSHE from *H. grisea* var. *thermoidea* and recombinantly expressed *H. grisea* var. *thermoidea* GSHE in *Trichoderma reesei*.

(1) pH stability was determined from pH 3 to 7.

10

The collected samples of recombinantly produced GSHE as described above and samples of native GSHE were diluted to equal protein concentrations with 20 mM acetate buffer at pH 4.5. Reactions were then run in 100mM citrate/NaOH buffers at 50°C for 30 minutes at pH levels 3 to 7.

15

1.0 ml of the reaction was then added to 5 ml of 10% corn starch (Cargill Foods, Minneapolis, MN) in 100mM acetate, pH 4.5 in sample tubes. The tubes were shaken at 50°C for 20 minutes. Then 0.5 ml 2.0% NaOH was added. Tubes were spun and 0.5 ml of the supernatant was assayed for reducing sugars using the Dinitro Salicylic acid (DNS) assay (Goto et al., (1994) *supra*, ).

20

The results of the assay are depicted in Figure 8A. The recombinantly produced GSHE exhibited about 80% residual activity at pH 3.5. In comparison, the corresponding native GSHE exhibited only about 20% residual activity. At pH 4.0 both the recombinantly produced GSHE and the native GSHE exhibited about 82% residual activity and at pH 5.5 both enzymes exhibited between about 90 to 100% residual activity.

25

Stability was also measured at pH 7.5 to 10.5 using the methods as described above. However, the buffer was 100mM boric acid /NaOH buffer. As exhibited in Figure 8B, at pH 7.5 both enzymes exhibited about 100% residual activity. At pH 8.5 recombinantly produced GSHE exhibited about 82% residual activity and the native GSHE exhibited about 90% residual activity. At pH 9.5 the % residual activity of recombinantly produced GSHE was substantially less than the native GSHE. (10% compared to 72%, respectively).

30

(2) Profile of activity as a function of temperature.

Temperature stability was determined at pH 5.75. Using essentially the same procedures as described above for the pH stability studies, enzyme samples were diluted to equal protein concentrations in a 100 mM acetate buffer and then 1.0 ml of the diluted

35

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enzymes was exposed to a water bath temperature of 40°C, 50°C, 60°C and 70°C for 10 minutes and assayed as described above in the pH stability studies. The results are presented in Table 5.

TABLE 5

GSHE Source	Temp °C	% Residual Activity
Native GSHE	40	100
	50	95
	60	90
	70	0
Recombinant GSHE	40	100
	50	93
	60	92
	70	0

% residual activity means the % difference referenced to 100% at pH 4.0

The profile of activity as a function of temperature of the recombinantly produced GSHE is similar to that of the corresponding native GSHE.

(3). Hydrolysis of granular corn starch by nGSHE and rGSHE.

Both native GSHE from *H. grisea* var. *thermoidea* (nGSHE) and recombinantly expressed *H. grisea* var. *thermoidea* (rGSHE) in *Trichoderma reesei* were diluted to equal protein concentrations in pH 4.5 acetate buffer. One ml of the dilution was added to a 10% corn starch (Cargill Foods, Minneapolis, MN) slurry in 20 mM pH 4.5 acetate buffer and shaken at 350 rpm at 50°C. At designated time intervals 100 µL of slurry was removed and added to 10 µL of 2% NaOH. The sample was spun and the supernatant was assayed for glucose (mg glucose/mg protein) using the glucose oxidase reagent in a Monarch clinical analyzer (Instrumentation Laboratory, Lexington, MA). As shown in Figure 9 the hydrolysis of corn starch was slightly lower for the rGSHE compared to the nGSHE.



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EXAMPLE 2

Expression of *Aspergillus awamori* var. *kawachi* GSHE gene in *Trichoderma reesei*.

A. Cloning the *Aspergillus awamori* var. *kawachi* GSHE gene

5 Genomic DNA was extracted from frozen mycelia of a strain of *A. awamori* var. *kawachi* according to the methods described in Example 1. The PCR primer sequences were designed based on the published sequence of the *A. awamori* var. *kawachi* glucoamylase GAI (Hayashida, *et al.* (1989) *Agric. Biol. Chem.* 53:923-929). This GAI is a GSHE. The following primers were used: the RSH10f primer having the sequence,  
10 CAC CAT GTC GTT CCG ATC TCT TCT C (SEQ ID NO:9), which includes the Gateway (Invitrogen) directional cloning motif CACC and the RSH11r primer having the sequence, CTA CCG CCA GGT GTC GGT CAC (SEQ ID NO:10).

The DNA sequence is provided in Figure 6 (SEQ ID NO:4). The encoded GSHE polypeptide sequence, including the signal peptide, is provided in Figure 7A (SEQ ID  
15 NO:5) and the mature protein sequence is provided in Figure 7B (SEQ ID NO:6).

The 2.16 kb PCR product was gel-purified (Gel Purification kit, Qiagen) and cloned into pENTR/D (Invitrogen), according to the Gateway system protocol. The vector was then transformed into chemically competent Top10 *E.coli* (Invitrogen) with kanamycin selection. Plasmid DNA from several clones was restriction digested to confirm the  
20 correct size insert. The GAI gene insert was sequenced (Sequetech, Mountain View, CA) from several clones (SEQ ID NO:4). Plasmid DNA from one clone, pENTR/D\_Ak33xx#1, was added to the LR clonase reaction (Invitrogen Gateway system) with the pTrex3g/*amdS* destination vector DNA. Recombination, in the LR clonase reaction, replaced the *Cm<sup>R</sup>* and *ccdB* genes of the destination vector with the *A. kawachi* GAI from  
25 the pENTR/D vector. This recombination directionally inserted GAI between the *cbhl* promoter and terminator of the destination vector. *AttB* recombination site sequences of 48 and 50 bp remained upstream and downstream, respectively, of the glucoamylase. Reference is made to Figure 3, wherein the *H. grisea gla1* has been replaced by the *A. kawachi* GAI in this example. Two microliters of the LR clonase reaction were  
30 transformed into chemically competent Top10 *E.coli* and grown overnight with carbenicillin selection. Plasmid DNA from several clones was digested with *XbaI* to confirm the insert size. Plasmid DNA from clone, pTrex3g\_Akxx #3 was digested with *XbaI* to release the expression cassette including the *cbhl* promoter:GAI:*cbhl* terminator:*amdS*. This 6.7 kb cassette was purified by agarose extraction using standard techniques and transformed  
35 into a strain of *T. reesei* derived from the publicly available strain QM6a.

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B. Transformation of *T. reesei* with the *A. awamori* var. *kawachi* GSHE gene

A *Trichoderma reesei* spore suspension was spread onto the center ~6 cm diameter of an MABA transformation plate (150  $\mu$ l of a  $5 \times 10^7$  -  $5 \times 10^8$  spore/ml suspension). The plate was then air dried in a biological hood. Stopping screens (BioRad 165-2336) and macrocarrier holders (BioRad 1652322) were soaked in 70% ethanol and air dried. DriRite desiccant was placed in small Petri dishes (6 cm Pyrex) and overlaid with Whatman filter paper. The macrocarrier holder containing the macrocarrier (BioRad 165-2335) was placed flatly on top of filter paper and the Petri dish lid replaced.

A tungsten particle suspension was prepared by adding 60 mg tungsten M-10 particles (microcarrier, 0.7 micron, Biorad #1652266) to an Eppendorf tube. One ml ethanol (100%) was added. The tungsten was vortexed in the ethanol solution and allowed to soak for 15 minutes. The Eppendorf tube was microfuged briefly at maximum speed to pellet the tungsten. The ethanol was decanted and washed three times with sterile distilled water. After the water wash was decanted the third time, the tungsten was resuspended in 1 ml of sterile 50% glycerol. The tungsten was prepared fresh every two weeks.

The transformation reaction was prepared by adding 25  $\mu$ l of suspended tungsten to a 1.5 ml Eppendorf tube for each transformation. Subsequent additions were made in order, 0.5-5  $\mu$ l DNA (0.2 -1  $\mu$ g/ $\mu$ l), 25  $\mu$ l 2.5M  $\text{CaCl}_2$ , 10  $\mu$ l 0.1 M spermidine. The reaction was vortexed continuously for 5-10 minutes, keeping the tungsten suspended. The Eppendorf tube was then microfuged briefly and decanted. The tungsten pellet was washed with 200  $\mu$ l of 70% ethanol, microfuged briefly to pellet and decanted. The pellet was washed with 200  $\mu$ l of 100% ethanol, microfuged briefly to pellet, and decanted. The tungsten pellet was resuspended, by pipetting, in 24  $\mu$ l 100% ethanol. The Eppendorf tube was placed in an ultrasonic water bath for 15 seconds and 8  $\mu$ l aliquots were transferred onto the center of the desiccated macrocarriers. The macrocarriers were left to dry in the desiccated Petri dishes.

A He tank was turned on to 1500 psi. 1100 psi rupture discs (BioRad 165-2329) were used in the Model PDS-1000/He Biolistic Particle Delivery System (BioRad). When the tungsten solution was dry, a stopping screen and the macrocarrier holder were inserted into the PDS-1000. An MABA plate, containing the target *T. reesei* spores, was placed 6 cm below the stopping screen. A vacuum of 29 inches Hg was pulled on the

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chamber and held. The He Biolistic Particle Delivery System was fired. The chamber was vented and the MABA plate removed for incubation, 28°C for 5-7 days.

With reference to Example 2 the solutions were prepared as follows.

5	<u>Modified <i>amdS</i> Biolistic agar (MABA)</u>	<u>per liter</u>
	Part I, make in 500 ml dH <sub>2</sub> O	
	1000x salts	1 ml
	Noble agar	20 g
10	pH to 6.0, autoclave	
	Part II, make in 500 ml dH <sub>2</sub> O	
	Acetamide	0.6 g
	CsCl	1.68 g
	Glucose	20 g
15	KH <sub>2</sub> PO <sub>4</sub>	15 g
	MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.6 g
	CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.6 g
20	pH to 4.5, 0.2 micron filter sterilize; leave in 50°C oven to warm, add to Part I, mix, pour plates.	
	<u>1000x Salts</u>	<u>per liter</u>
	FeSO <sub>4</sub> · 7H <sub>2</sub> O	5 g
	MnSO <sub>4</sub> · H <sub>2</sub> O	1.6 g
25	ZnSO <sub>4</sub> · 7H <sub>2</sub> O	1.4 g
	CoCl <sub>2</sub> · 6H <sub>2</sub> O	1 g
	0.2 micron filter sterilize	

30 Expression of rGSHE (*A. awamori* var. *kawachi* GSHE expressed in *T. reesei*) was determined as described above for expression of *H. grisea* var. *thermoidea* in Example 1. The level of expression was determined to be greater than 1 g/L (data not shown). Figure 10 provides the results of a SDS-PAGE gel illustrating the expression of *Aspergillus*  
 35 *awamori* var. *kawachi* GSHE in the *T. reesei* host.

### EXAMPLE 3

Solubilization and hydrolysis of different granular starch substrates by alpha amylase.

40 In a typical experiment, 150 grams of granular starch were suspended in 350 grams of distilled water. After mixing, the pH was adjusted to pH 5.5 using 6 N NaOH. The alpha amylase (GZYME G997 at 1.0kg/MT of starch, ds) was added to the starch slurry and incubated with constant stirring in a water bath maintained at 60°C. The samples were

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withdrawn at different time intervals for measuring the Brix. The sample withdrawn at 24 hrs was used to determine the sugar composition using HPLC (Table 6).

TABLE 6

G Zyme G 997 (0.1 kg/MT starch)	% Solids solublized Incubation time (hr)						% Carbohydrate composition at 24 hr			
	2	4	6	9	12	24	DPI	DP2	DP3	DP4 <sup>+</sup>
Corn	32.2	39.3	42.9	49.2	52.8	53.9	0.6	12.0	15.0	72.4
Tapioca	46.6	50.8	53.6	56.1	58.2	62.4	1.6	11.6	14.6	72.2
Wheat	74.9	80.6	82.4	84.5	86.3	87.8	1.3	11.5	14.1	73.1

- 5 The results illustrated in Table 6 show significant differences in the solubilization of the granular starch substrates. Wheat had the highest % of solublized solids, and corn had the lowest percent. Significant differences were not observed in the sugar composition after 24 hours.

#### EXAMPLE 4

- 10 Solubilization and hydrolysis of granular starch substrates by  
alpha amylase (G ZYME G 997) and rH-GSHE.

- In a typical experiment, 350 g of water was added separately to 150 grams of each, granular cornstarch, granular wheat starch and granular tapioca starch and the pH was  
15 adjusted to pH 5.5 using 6N NaOH. The slurry was kept in a water bath, which was maintained at 60°C with continuous stirring for uniform mixing. After stabilization of the temperature, alpha amylase as G Zyme G 997 (0.1Kgs/MT of starch ds) and rH-GSHE (1.0 GSHE Units/gram of starch ds) were added to each starch slurry and incubation was continued. Samples were taken at different intervals of time, centrifuged and Brix was  
20 checked. The 24 hour samples were analyzed for sugar composition. The relative solubilization of the granular starch was calculated by comparing the Brix from the jet cooking process and reference is made to Table 7.

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TABLE 7

Starch	% Relative Solubilization (hrs)						% Soluble Sugar (24 hrs)			
	2	4	6	12	18	24	DP1	DP2	DP3	DP4+
Wheat	86.0	91.0	93.9	95.7	97.5	98.2	97.3	2.3	0.2	0.2
Corn	61.3	78.4	87.9	98.2	100.0	100.0	97.4	2.1	0.2	0.2
Tapioca	64.9	75.4	81.8	87.4	91.6	97.5	96.5	1.6	0.4	1.5

The combined effect of G Zyme G 997 and rH-GSHE resulted in almost complete solubilization of the granular starch substrate under mild conditions compared to the current high temperature jet cooking process. The analysis of the 24-hour samples showed glucose yield greater than 96.5%.

EXAMPLE 5

Solubilization and hydrolysis of granular cornstarch by glucoamylases having granular starch hydrolyzing activity.

Commercially available glucoamylases exhibiting granular starch hydrolyzing activity from *Aspergillus niger* (OPTIDEX L-400 and G Zyme G 990 4X from Genencor International Inc), and *Rhizopus niveus* (CU. CONC. from Shin Nihon Chemicals, Japan) were compared with rH-GSHE as described above in example 1. The granular starch hydrolyzing activity of these products was measured using the assay described above.

TABLE 8

Glucoamylase	GSHE units/g
OPTIDEX L-400	555
G Zyme G 990 4X	474
CU.CONC.	1542
rH-GSHE	518

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In a typical experiment, a 30% granular cornstarch slurry in distilled water (150 grams of starch in 350 grams of distilled water) was prepared and the pH was adjusted to pH 5.5 using 6 N NaOH. G Zyme G 997 was added at 0.1 kg/MT of starch ds and the starch slurry was kept in a water bath maintained at 60 °C. To each starch slurry containing G Zyme G 997, different glucoamylases were added at an equal dosage, i.e.; 1.5 GSHE units /gram starch, ds and incubated at 60°C. An aliquot was withdrawn at different time intervals and centrifuged. The clear supernatant was used for measuring the Brix. The sample incubated for 2, 6, 22.5 and 49 hours was analyzed by HPLC for total sugar composition and the results are shown in Table 9.

TABLE 9

Enzyme	Hr	% DP1	% DP2	% DP3	% DP4+	% Solubilized
Distillase L-400	2	94.2	0.9	0.2	4.7	
G990 4X	2	95.9	0.7	0.2	3.2	
Cu Conc	2	73.5	11.1	1.4	14.0	
rH-GSHE	2	96.4	1.1	0.1	2.3	
Distillase L-400	6	96.1	1.2	0.2	2.5	
G990 4X	6	96.7	1.4	0.2	1.7	
Cu Conc	6	79.1	8.0	1.0	11.8	
rH-GSHE	6	97.9	1.4	0.0	0.7	
Distillase L-400	22.5	96.8	2.1	0.2	0.9	
G990 4X	22.5	96.6	2.5	0.2	0.7	
Cu Conc	22.5	81.9	6.5	1.1	10.5	
rH-GSHE	22.5	96.2	3.4	0.2	0.1	
Distillase L-400	49	96.3	3.0	0.2	0.5	<b>81.6</b>
G990 4X	49	96.0	3.3	0.2	0.4	<b>81.6</b>
Cu Conc	49	80.8	6.9	1.5	10.8	<b>74.6</b>
rH-GSHE	49	93.8	5.6	0.5	0.1	<b>97.5</b>

Glucoamylases were added at a dose of 1.5 GSHEU/g to a starting slurry having 0.1KG/MT ds of alpha amylase.

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EXAMPLE 6

The effect of pH on the solubilization of granular cornstarch (35% slurry) during incubation with alpha amylase (G Zyme G 997) and rH-GSHE.

In a typical experiment, 372 grams of water was added to 178 grams of cornstarch. The slurry was stirred well for uniform mixing and the pH of the slurry was adjusted to pH 4.0, 5.0, 5.5, 6.0, 6.5 and 7.0 using 6 N NaOH. The samples were then kept in a water bath maintained at 60 °C. After equilibration of the temperature, Zyme G997 at 0.1 kg/MT starch and rH-GSHE as described in example 1 (1.0 GSHE Units/g starch) were added to the slurry. The slurry was continuously stirred during incubation and samples were taken after one hour for measuring the brix (Table 10).

TABLE 10

Incubation pH at 60°C	% Maximum Solubilization
4.0	9.9
5.0	100.0
5.5	100.0
6.0	95.0
6.5	92.0
7.0	76.7

The maximum solubilization occurred at pH 5.0 and pH 5.6. A significant reduction in the solubilization of the granular cornstarch occurred below pH 5.0 and pH 5.5 at 60 °C indicating either lower activity or inactivation of the enzymes.

EXAMPLE 7

Effect of temperature on the solubilization of the granular cornstarch (32% slurry) during incubation with alpha amylase and rH-GSHE.

In a typical experiment, 372 grams of water was added to 178 grams of cornstarch. The slurry was stirred well for uniform mixing, and the pH of the slurry was adjusted to pH 5.5, using 6 N NaOH. The samples were kept in a water bath maintained at 55°C, 60°C and 65°C. After equilibration of the temperature, Zyme G997 at 0.1 Kgs/MT starch and rH-

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GSHE as described in example 1 (1.0 GSHE Units/g starch) were added. The slurry was continuously stirred during incubation and the brix was measured after one hour. (Table 11).

TABLE 11

Incubation Temperature °C	% Starch Solubilized
55	28.7
60	51.4
65	59.6
70	75.1

The solubility of the granular cornstarch was increased with increasing temperature in the presence of G Zyme G997 and rH-GSHE. However HPLC analysis of the solubilized carbohydrate above 65 °C indicated inactivation of rH-GSHE as evidenced by lower level  
of glucose content. The increase in the dissolved solids content at higher temperature  
(>65° C) was mainly due to the liquefaction effect of G Zyme G997 on granular cornstarch at higher temperatures.

#### EXAMPLE 8

Effect of G Zyme G997 and rH-GSHE concentrations on the solubilization and hydrolysis of granular cornstarch.

In different flasks granular cornstarch (178g) in 372 g water was stirred well for uniform mixing. The pH of the slurry was adjusted to pH 5.5. Two different levels of G Zyme G 997, 0.1 Kgs/MT starch and 0.5 Kgs/MT were incubated with rH-GSHE at 0.25, 0.5, and 1.0 GSHE units/g ds starch at 60°C. Samples were drawn at different intervals time, and used for measuring the brix and total sugar composition (Tables 12A and 12B).



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TABLE 12A

Enzyme Concentration		% Relative Solubilization					
G Zyme G997 Kgs/MT starch	rH-GSHE GSHE Units/g starch	3hr	6hr	9hr	12hr	24hr	30hr
0.1	0.25	50.9	59.3	70.2	75.1	85.8	88.6
0.1	0.50	54.3	72.9	80.4	85.2	94.0	96.8
0.1	1.0	60.7	80.4	88.6	92.1	98.1	100
0.5	0.25	60.9	71.9	77.6	83.3	91.2	94.0
0.5	0.5	66.9	79.5	85.8	89.9	97.2	99.4
0.5	1.0	76.7	87.4	92.4	95.3	99.1	99.7

The results in Table 12A indicate that increasing the dosage of G Zyme G 997  
 5 from 0.1 Kgs/ MT of starch to 0.5 Kg/MT of starch resulted in a faster solubilization of  
 granular starch. But at both levels greater than 95% solubilization of granular starch  
 occurred in 24 hours in presence of 1.0 GSHE units of rH-GSHE. The effect of rH-GSHE  
 concentration on the solubilization of granular starch in the presence of G Zyme G 997  
 increased dramatically with increasing dosage of concentration. The above results clearly  
 10 show that neither of the enzymes, G Zyme G 997 or rH-GSHE alone can solublize the  
 granular starch to completion. However, complete solubilization of the granular starch  
 occurred when the enzymes were added together.

The carbohydrate (sugar) composition of the solublized granular (32% slurry)  
 cornstarch during incubation of the granular cornstarch with G ZYME G997 and rH-GSHE  
 15 at 12, 24 and 30 hour at pH 5.5 and 60°C was analyzed by HPLC and reference is made  
 to Table 12B.

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TABLE 12B

Enzyme composition G Zyme G997      rH-GSHE kg/MT st          GSHE U/g st		Incubation Time (hr)	% starch solubilized	% Carbohydrate Composition			
DP1	DP2	DP3	DP4 <sup>+</sup>				
0.1	0.25	12	75.1	87.9	4.1	0.9	7.2
		24	85.8	93.2	2.4	0.8	3.6
		30	88.6	94.7	2.0	0.8	2.6
0.1	0.50	12	85.2	95.9	1.6	0.4	2.1
		24	94.0	97.0	1.8	0.3	0.9
		30	96.8	97.5	2.0	0.2	0.4
0.1	1.00	12	92.1	97.5	1.9	0.2	0.5
		24	98.1	96.9	2.8	0.2	0.2
		30	100.0	96.3	3.3	0.3	0.2
0.5	0.25	12	83.3	84.4	6.4	1.4	7.8
		24	91.2	92.0	3.0	1.3	3.6
		30	94.0	94.0	2.3	1.1	2.6
0.5	0.50	12	98.9	95.0	2.0	0.9	2.4
		24	97.2	96.6	1.9	0.5	0.9
		30	99.4	96.9	2.1	0.4	0.6
0.5	1.00	12	95.3	97.1	1.9	0.3	0.7
		24	99.1	96.7	2.3	0.3	0.2
		30	99.7	96.4	3.2	0.3	0.2

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The results in Table 12B illustrate that an appropriate blend of *Bacillus* *stearothermophilus* alpha amylase and rH-GSHE would meet a variety of demands in the commercial production of sugar sweeteners and biochemicals directly from granular starch without applying conventional high temperature cooking process. High levels of alpha  
5 amylase accelerates the rate of solubilization of granular starch but higher level of rH-GSHE resulted in high levels of reversion reaction products resulting in significantly low levels of higher sugar.

#### 10 EXAMPLE 9

Comparison on the hydrolysis of enzyme liquefied cornstarch substrate (soluble starch substrate) and granular cornstarch substrate (insoluble) by glucoamylase preparations.

In a typical experiment, cornstarch (32 % ds) was liquefied at pH 5.6 using the low temperature jet cooking process (105°C, 8 min) followed by hydrolysis at 95°C for 90 min.  
15 SPEZYME FRED (Genencor International Inc) was added at 0.4 Kgs/MT of starch, ds. in the liquefaction process. The pH of the SPEZYME FRED liquefied starch substrate was adjusted to pH 4.2 and glucoamylase (OPTIDEX L-400) was added at 0.22GAU/g ds. The hydrolysis was carried out at 60°C. Samples were withdrawn at different time intervals and analyzed by HPLC to determine the time required for reaching the maximum glucose  
20 yield.

Thirty two percent ds granular cornstarch slurry in distilled water was prepared and the pH of the slurry was adjusted to pH 5.5 using 1 N NaOH. The flask was then kept in a water bath maintained at 60°C and G Zyme G997 was added at 0.1 Kgs/MT ds and rH-GSHE was added at 1.0 GSHE Units/gram ds and the sample was incubated at 60°C with  
25 constant stirring. Samples were withdrawn at different intervals of time for measuring the BRIX and glucose yield (Table 13).

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TABLE 13

Substrate	Glucoamylase	Time (hr) for reaching Max. glucose	Composition at Maximum. Glucose yield (%)			
			DP1	DP2	DP3	DP4 <sup>+</sup>
Liquefied Starch Soluble, 32% slurry	OPTIDEX L-400	61	95.2	3.1	0.4	1.3
Granular Starch Insoluble, 32% slurry	rH-GSHE and G ZYME G997	24	96.8	2.8	0.2	0.2

Hydrolysis of the liquefied soluble starch by glucoamylase required a longer time to achieve the maximum glucose yield compared to the hydrolysis of insoluble granular starch. At the peak time for reaching maximum glucose yield, the glucose level by granular starch as compared to liquefied soluble starch was higher with significantly lower sugars at DP4<sup>+</sup> (96.8 and 0.2 compared with 95.2 and 1.3).

Higher glucose yield, the potential for shorter saccharification time and a total elimination of high temperature jet cooking step, differentiates the application of the alpha amylase and GSHE enzyme blend of the present invention.

#### EXAMPLE 10

Effect of granular cornstarch concentration on the solubilization and hydrolysis of starch during the incubation with G Zyme G 997 (0.1 Kgs/MT) and rH-GSHE (1.0 GSHE Units/g).

Different concentrations of granular cornstarch slurry were prepared in distilled water. i.e., 32%, 35%, 38%, 40% and 42%. The pH of the slurry was adjusted to pH 5.5. The samples were then kept in a water bath maintained at 60°C and stirred continuously for uniform mixing. G Zyme G997 (0.1 Kgs/MT of ds) and rH-GSHE obtained from Example 1 (1.0 GSHE units/g ds) were added to the slurry. An aliquot sample was withdrawn at different time intervals during incubation for measuring brix and sugar composition (Table 14).

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TABLE 14

Trial	Starting % DS	Sample % DS	Hours	% Cabohydrate Profile			
				DP>3	DP3	DP2	DP1
1	32	0.41	0				
1	32	21.31	2.5				
1	32	26.84	7	1.56	0.35	1.71	96.38
1	32	33.55	24	0.28	0.39	3.30	96.03
1	32	34.50	48	0.49	0.40	3.83	95.28
2	35	0.49	0				
2	35	22.57	2.5				
2	35	28.57	7	1.24	0.35	1.81	96.60
2	35	34.50	24	0.33	0.71	4.16	94.80
2	35	36.91	48	0.21	0.68	4.71	94.40
3	38	0.62	0				
3	38	23.89	2.5				
3	38	29.67	7	1.38	0.33	1.84	96.45
3	38	35.76	24	0.31	0.38	3.21	96.11
3	38	38.64	48	0.48	0.56	4.76	94.19
4	40	0.34	0				
4	40	27.78	2.5				
4	40	30.47	7	1.36	0.35	1.94	96.35
4	40	36.58	24	0.38	0.50	2.79	96.33
4	40	39.71	48	0.22	0.70	4.77	94.31
5	42	0.48	0				
5	42	25.31	2.5				
5	42	31.64	7	1.23	0.34	2.00	96.43
5	42	37.94	24	0.63	0.35	3.09	95.93
5	42	40.82	48	0.40	1.29	6.57	91.74

The results show over 96% glucose syrup could be reached within 24 hours of the saccharification at dissolved solids as high as 36%. The glucose yield of greater than 96% at a solid level higher than 35% was reached when an insoluble granular starch was used in the saccharification process.

#### EXAMPLE 11

As taught above and known in the art, the enzyme-enzyme starch conversion process for high glucose syrup consists of producing a soluble liquefact by subjecting an insoluble starch substrate to a high temperature liquefaction process using a thermostable alpha amylase. It is the normal practice in the commerce to inactivate the residual alpha amylase activity prior to the saccharification by glucoamylase to reduce the loss of glucose

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yield due to the presence of active alpha amylase. The inactivation of the residual alpha amylase activity is normally carried out by decreasing the liquefact to pH 4.2 at 95° C. High temperature and pH 4.5 result in the complete inactivation of the alpha amylase. So we studied the effect of glucose yield with and without active alpha amylase during  
 5 saccharification of liquefact starch at pH 5.5.

In a typical experiment, soluble liquefact from cornstarch was produced using G Zyme G 997 (0.4 Kgs / MT starch) as a liquefying enzyme under jet cooking process conditions (32% ds starch at 105°C at 8 min followed by 95°C for 90 min). A portion of the liquefied starch was further heated to inactivate the residual alpha amylase activity. The  
 10 saccharification of the liquefied starch with and without residual alpha amylase activity was further saccharified (32% ds) at pH 5.5, 60 °C using rH-GSHE as described in example 1 at 0.5 GSHE units/g. Samples were withdrawn at different intervals of time and analyzed for glucose yield using HPLC (Table 15).

TABLE 15

15	Alpha Amylase Activity		Sac. Time (Hrs)	% Sugar Composition			
	During Saccharification	Substrate		DP1	DP2	DP3	DP4 <sup>+</sup>
20	Inactive	Soluble	18	91.48	1.85	0.41	6.25
			24	94.37	1.88	0.33	3.43
		Liquefact	42	96.08	2.74	0.32	0.86
			48	96.01	2.95	0.30	0.74
			68	95.25	3.79	0.44	0.52
25	Active	Soluble	18	91.88	2.54	1.19	4.39
			24	94.31	2.20	1.00	2.49
		Liquefact	42	95.78	2.84	0.62	0.77
			48	95.85	3.03	0.53	0.59
			68	95.33	3.85	0.57	0.24
30	Active	Insoluble starch	24	96.84	2.75	0.23	0.18

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The results in Table 15 demonstrate that the presence of alpha amylase activity of G Zyme G 997 during the saccharification of the soluble starch substrate (liquefact) by glucoamylase resulted in the lower glucose yield. Whereas, alpha amylase enhances the hydrolysis of insoluble (granular) starch substrate by glucoamylase resulting in a substantially high level of glucose.

### EXAMPLE 12

Production of glucose syrup and residual starch from hydrolysis of corn starch

In a reactor vessel, granular cornstarch (800 g) in 1200 g water was stirred well for uniform mixing to obtain a slurry. The pH of the slurry was adjusted to pH 5.5 with 4 N NaOH. G ZYME G 997 (0.1 Kgs/MT starch) and *Humicola grisea* var. *theromidea* expressed in *Trichoderma reesei* ( rH-GSHE) at 1.0 GSHE U/g starch were added at 60°C. Samples were withdrawn at various time intervals and used for measuring the brix and total sugar composition (Table 16).

Table 16

Reaction Time	3 hr	6 hr	9 hr
% Solubilization	60.7	80.4	88.6

After achieving greater than 90% solubilization of granular starch in a 10 hour reaction time, the sugar composition, measured by HPLC, of the solubilized granular cornstarch was achieved as illustrated in Table 17.

Table 17

Sugar Type	DP1	DP2	DP3	DP 4+
% Composition	97.5	1.9	0.2	0.5

After a 10 hr reaction time, the hydrolysis was stopped by adjusting the pH to 3.5 with 4N H<sub>2</sub>SO<sub>4</sub>. The syrup mixture contained greater than 96% dextrose at greater than 30% dissolved solids. The mixture was filtered at 60°C by using a 500,000 molecular weight cut-off (MWCO) ultrafiltration membrane cartridge (AG Technology, MA). The

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A sample of the residual starch was dried and examined for its structure by scanning electron microscope (Hitachi S-5000 cold field emission SEM (Tokyo, Japan)) and compared with a typical starch granular before expose to enzymes in accordance with the method of the invention (Figure 12). The dried samples were mounted on SEM sample  
5 stubs using double-sided adhesive carbon tape. Any excess sample was removed by dusting with compressed air. The samples were then mounted at about a 30° angle in a Bal-Tec Med 020 Modular High Vacuum Coating System (Liechtenstein) and sputter coated with 10 nm of platinum (Pt) while rotating at 60 rpm. These settings ensured that the granules were evenly coated on all sides. The thickness of the coating is negligible  
10 compared to the size of the features resulting from the enzymatic action of the starch granules. The accelerating voltage varied from 2 – 5kV and magnification was between 500 – 15,000x.

Micrograph Fig.12a depicts a typical starch granule before exposure to an enzyme composition and method of the invention. The surface is smooth and homogenous and the  
15 only noticeable feature is a fine cracking due to the platinum metal coating. Once exposed to the enzyme blend and method of the invention, the surface morphology of the granules change. As seen in micrographs b – d of figure 12, large round holes are bored into the granules due to enzyme digestion of the starch granule substrate. The holes range in diameter and vary in depth, and reflect a population of starch granules at different kinetic  
20 stages of enzymatic reaction. Some granules have only a few number of holes and some are nearly covered in holes (micrograph b). Some granules were also sliced in half revealing a cross section of digested interior (micrographs c and d). Micrograph d) reveals granule digestion to completion, showing a fragment of a hollowed out shell.



## CLAIMS

1. A method for producing a granular starch hydrolyzing enzyme having glucoamylase activity (GSHE) in a filamentous fungal host cell comprising
  - a) transforming a filamentous fungal host cell with a DNA construct comprising a promoter having transcriptional activity in the filamentous fungal host cell operably linked to a heterologous polynucleotide encoding a GSHE,
  - b) cultivating the transformed filamentous fungal host cell in a suitable culture medium to allow expression of said GSHE, and
  - c) producing the GSHE.
2. The method according to claim 1 further comprising recovering the produced GSHE.
3. The method according to claim 1, wherein the heterologous polynucleotide encoding the GSHE is derived from a strain of *Humicola grisea* or a strain of *Aspergillus awamori*.
4. The method according to claim 3, wherein the GSHE has at least 90% sequence identity with the sequence of SEQ ID NO: 3 or SEQ ID NO: 6.
5. The method according to claim 1, wherein the filamentous fungal host cell is a *Trichoderma* cell.
6. The method according to claim 1, wherein a level of glycosylation of the expressed GSHE from the transformed filamentous fungal host cell is less than the level of glycosylation of the GSHE expressed in a native fungal host.
7. The method according to claim 1, wherein enzyme activity at a pH level of 3.0 to 4.0 is greater for the produced GSHE in the transformed filamentous fungal host cell than the corresponding produced GSHE from a native fungal host.
8. The method according to claim 1, wherein the transformed fungal host cell is cultured under continuous fermentation conditions.

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9. The method according to claim 1, wherein the transformed fungal host is cultured under batch fermentation conditions.

10. A recombinant *Trichoderma* cell comprising a heterologous polynucleotide encoding a granular starch hydrolyzing enzyme having glucoamylase activity (GSHE).

11. The recombinant *Trichoderma* cell of claim 10, wherein said heterologous polynucleotide is derived from a strain of *Humicola grisea* or from a strain of *Aspergillus awamori*.

12. A fermentation medium comprising a granular starch hydrolyzing enzyme having glucoamylase activity (GSHE) produced from a culture of *Trichoderma reesei*, wherein the *Trichoderma reesei* comprises a heterologous polynucleotide encoding a GSHE having at least 90% amino acid sequence identity with SEQ ID NO: 3 or with SEQ ID NO: 6.

13. A process for producing a glucose syrup from a granular starch slurry, said process comprising contacting a granular starch substrate simultaneously with the fermentation medium of claim 12 and an alpha amylase derived from a *Bacillus* strain at a temperature below the gelatinization temperature of the granular starch and allowing the alpha amylase and GSHE to act for a period of time between 2 and 100 hours to hydrolyze the granular starch to obtain a glucose syrup.

14. An enzyme composition having granular starch hydrolyzing activity comprising an alpha amylase and a glucoamylase having granular starch hydrolyzing activity (GSHE), wherein the ratio of the alpha amylase to GSHE is between 15 : 1 and 1 : 15.

15. The enzyme composition of claim 14, wherein the alpha amylase to GSHE ratio is 10:1 to 1:10.

16. A one-step process for producing a glucose syrup from a granular starch slurry, said process comprising

contacting a granular starch slurry obtained from a granular starch substrate simultaneously with an alpha amylase and a glucoamylase having

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granular starch hydrolyzing activity (GSHE) at a temperature equal to or below the gelatinization temperature of the granular starch and

allowing the alpha amylase and the GSHE to act for a period of time sufficient to hydrolyze the granular starch to obtain a composition comprising a glucose syrup.

17. The process according to claim 16, wherein the granular starch substrate is obtained from corn, wheat, barley or rice.

18. The process according to claim 17, wherein the granular starch substrate is corn starch obtained from whole grain.

19. The process according to claim 16, wherein the granular starch substrate is dry milled or wet milled.

20. The process according to claim 16, wherein the granular starch slurry has a dry solids content of 15 – 55%.

21. The process according to claim 20, wherein the granular starch slurry has a dry solids content of 15 – 45%.

22. The process according to claim 16, wherein the temperature is less than 65°C and greater than 50°C.

23. The process according to claim 22, wherein the temperature is less than 65°C and greater than 55°C.

24. The process according to claim 16, wherein the period of time sufficient to hydrolyze the granular starch is 2 to 100 hours.

25. The process according to claim 16, wherein the contacting step is conducted at a pH of 5.0 to 6.0.

26. The process according to claim 16, wherein the alpha amylase is derived from a *Bacillus*.

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27. The process according to claim 16, wherein the amount of alpha amylase per metric ton of starch is in the range of 0.05 to 5.0 kg.

28. The process according to claim 27, wherein the amount of alpha amylase per metric ton of starch is in the range of 0.05 to 2.0 kg.

29. The process according to claim 16, wherein the GSHE is obtained from the heterologous expression of a polynucleotide encoding a GSHE having at least 90% sequence identity to the sequence of SEQ ID NO: 3 or SEQ ID NO: 6.

30. The process according to claim 16, wherein the amount of GSHE is in the range of 0.01 to 10.0 GSHE U/g starch dry solids.

31. The process according to claim 30, wherein the amount of GSHE is in the range of 0.1 to 5.0 GSHE Units/g starch dry solids.

32. The process according to claim 31, wherein the amount of GSHE is in the range of 0.25 to 2.5 GSHE Units/g starch dry solids.

33. The process according to claim 16, wherein at least 90% of the granular starch is hydrolyzed within 24 hours.

34. The process according to claim 16 further comprising recovering the glucose syrup.

35. The process according to claim 34, wherein the glucose syrup is recovered by filtration separation.

36. The process according to claim 16 further comprising converting the glucose syrup to a desired end product.

37. The process according to claim 36, wherein the desired end product is a fructose syrup.

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38. The process according to claim 36, wherein the desired end product is ethanol.
39. The process according to claim 36, wherein the desired end product is a crystallized glucose.
40. The process according to claim 35, wherein residual starch is obtained as a by product of the glucose syrup separation.
41. The process according to claim 40 further comprising recycling the residual starch by contacting the residual starch with the GSHE and alpha amylase at a temperature below the gelatinization temperature of the granular starch.
42. The residual starch obtained according to the process of claim 40.
43. A process for the production of a high fructose starch-base syrup comprising enzymatically converting the glucose syrup obtained according to the process of claim 16 to a fructose syrup.
44. The process according to claim 43, wherein the converting step includes use of a glucose isomerase.
45. A process for production of a residual starch composition comprising, contacting a granular starch substrate according to claim 16 to produce a composition comprising a glucose syrup and separating the glucose syrup from the composition to obtain a mixture including residual starch.
46. A method of using the residual starch obtained according to claim 45 in a starch saccharification processes.
47. A process for the production of ethanol comprising subjecting the glucose syrup produced according to claim 16 to fermentation with an ethanolgenic microorganism.
48. The process according to claim 47, wherein the fermentation is carried out separately and sequentially to the contacting step.

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49. The process according to claim 47, wherein the fermentation is carried out simultaneously with the contacting step.

50. The process according to claim 47 further comprising recovering the ethanol from the fermentation.

Figure 1. *H. grisea* GSHE nucleotide sequence with *putative* introns bold & underlined.

ATGCATACCTTCTCCAAGCTCCTCGTCCTGGGCTCTGCCGTCCAGTCTGCCCTCGGGCGGCCTCACGGCT  
 CTTTCGCGTCTCCAGGAACGCGCTGCCGTTGATACCTTCATCAACACCGAGAAGCCCATCGCATGGAACAA  
 GCTGCTCGCCAACATCGGCCCTAACGGCAAAGCCGCTCCCGGTGCCGCCGCGCGGCGTTGTGATTGCCAGC  
 CCTTCCAGGACGGACCCTCCTTGTACGTGGTGGCATGGAATGGACCCAGAGACTGGTTTTAGATGAAAG  
AGAGTTTCTGCTAACCGCCACACCCAGACTTCTTCACTGGACCCGCGATGCCGCCCTGGTCTCACCGG  
 CATCATCGAGTCCCTTGGCCACAAC TACAACACCACCCTGCAGACCGTCATCCAGAACTACGTCGCGTCG  
 CAGGCCAAGCTGCAGCAGGTCTCGAACCCCTCGGGAACCTTCGCCGACGGCTCGGGTCTCGGTGAGGCCA  
 AGTTCAATGTGACCTCACTGCCTTCACTGGCGAATGGGGTCCGCCCTCAGAGGGACGGCCCGCCCTGCG  
 CGCCATCGCTCTCATCCAGTACGCCAAGTGGCTGATCGCCAACGGCTACAAGAGCACGGCCAAGAGCGTC  
 GTCTGGCCCGTCTCAAGAACGATCTCGCCTACACGGCCAGTACTGGAACGAGACCGGCTTCGATCTCT  
 GGGAGGAGGTCCCCGGCAGCTCGTTCCTTACCATCGCCAGCTCTCACAGGGGTGAGTCA TTTATTGTTCA  
GTGTTTCTCATTGAATAATTACCGGAATGCCACTGACGCCAAACAGCTCTGACTGAGGGTGCTTACCTC  
 GCCGCTCAGCTCGACACCGAGTGCCGCGCCTGCACGACCGTCGCCCCCTCAGGTTCTGTGCTTCCAGCAGG  
 CCTTCTGGAACCTCCAAGGGCAACTATGTCGTCTCCAACAGTAAGATCCCTACACCAACAAAAAATCGA  
AAAGGAACGTTAGCTGACCTTCTAGTCAACGGCGGCGAGTATCGTCCGGCAAGGACGCCAACTCGATC  
 CTGGCGTCCATCCACAAC TTCGACCCTGAGGCCGGCTGCGACAACCTGACCTTCCAGCCCTGCAGCGAGC  
 GCGCCCTGGCCAACCACAAGGCCTATGTCGACTCGTTCGCAACCTCTACGCCATCAACAAGGGCATCGC  
 CCAGGGCAAGGCCGTTGCCGTCGGCCGCTACTCGGAGGATGTCTACTACAACGGCAACCCGTGGTACCTG  
 GCCAACTTTGCCGCCGCCGAGCAGCTCTACGACGCCATCTACGTGTGGAACAAGCAGGGCTCCATCACCG  
 TGACCTCGGTCTCCCTGCCCTTCTTCCGCGACCTTGTCTCGTCCGTGAGCACCGGCACCTACTCCAAGAG  
 CAGCTCGACCTTCACCAACATCGTCAACGCCGTCAAGGCCTACGCCGACGGCTTCATCGAGGTGGCGGCC  
 AAGTACACCCCGTCCAACGGCGCGCTCGCCGAGCAGTACGACCGCAACACGGGCAAGCCCGACTCGGCCG  
 CCGACCTGACGTGGTCTGACTCGGCCCTTCTCTCGGCCATCGACCGCCGCGCGGGTCTCGTCCCCCGAG  
 CTGGCGGGCCAGCGTGGCCAAGAGCCAGCTGCCGTCCACCTGCTCGCGCATCGAGGTGCGCGGCACCTAC  
 GTCGCCGCCACGAGCACCTCGTTCCCGTCCAAGCAGACCCCGAACCCCTCCGCGGCGCCCTCCCGTCCC  
 CCTACCCGACCGCTGCGCGGACGCTAGCGAGGTGTACGTACCTTCAACGAGCGCGTGTGACCGCGTG  
 GGGCGAGACCATCAAGGTGGTGGGCAACGTGCCGGCGCTGGGGAAC TGGGACACGTCCAAGGCGGTGACC  
 CTGTCCGCCAGCGGGTACAAGTCGAATGATCCCTCTGGAGCATCACGGTGCCCATCAAGGCGACGGGCT  
 CGGCCGTGCAGTACAAGTATATCAAGGTCCGCACCAACGGGAAGATTACTTGGGAGTCGGACCCCAACAG  
 GAGCATTACCCTGCAGACGGCGTCTCTCGGGCAAGTGCGCCGCGCAGACGGTGAATGATTCTGCGCGT  
 TAA

Figure 2A. *H. grisea* GSHE protein sequence with *putative* signal sequence underlined.

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M H T F S K L L V L G S A V Q S A L G R P H G S S R L Q E R A A V D T F I N T
E K P I A W N K L L A N I G P N G K A A P G A A A G V V I A S P S R T D P P Y
F F T W T R D A A L V L T G I I E S L G H N Y N T T L Q T V I Q N Y V A S Q A
K L Q Q V S N P S G T F A D G S G L G E A K F N V D L T A F T G E W G R P Q R
D G P P L R A I A L I Q Y A K W L I A N G Y K S T A K S V V W P V V K N D L A
Y T A Q Y W N E T G F D L W E E V P G S S F F T I A S S H R A L T E G A Y L A
A Q L D T E C R A C T T V A P Q V L C F Q Q A F W N S K G N Y V V S N I N G G
E Y R S G K D A N S I L A S I H N F D P E A G C D N L T F Q P C S E R A L A N
H K A Y V D S F R N L Y A I N K G I A Q G K A V A V G R Y S E D V Y Y N G N P
W Y L A N F A A A E Q L Y D A I Y V W N K Q G S I T V T S V S L P F F R D L V
S S V S T G T Y S K S S S T F T N I V N A V K A Y A D G F I E V A A K Y T P S
N G A L A E Q Y D R N T G K P D S A A D L T W S Y S A F L S A I D R R A G L V
P P S W R A S V A K S Q L P S T C S R I E V A G T Y V A A T S T S F P S K Q T
P N P S A A P S P S P Y P T A C A D A S E V Y V T F N E R V S T A W G E T I K
V V G N V P A L G N W D T S K A V T L S A S G Y K S N D P L W S I T V P I K A
T G S A V Q Y K Y I K V G T N G K I T W E S D P N R S I T L Q T A S S A G K C
A A Q T V N D S W R

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Figure 2B. *H. grisea* Mature GSHE protein sequence

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A A V D T F I N T E K P I A W N K L L A N I G P N G K A A P G A A A G V V I A
S P S R T D P P Y F F T W T R D A A L V L T G I I E S L G H N Y N T T L Q T V
I Q N Y V A S Q A K L Q Q V S N P S G T F A D G S G L G E A K F N V D L T A F
T G E W G R P Q R D G P P L R A I A L I Q Y A K W L I A N G Y K S T A K S V V
W P V V K N D L A Y T A Q Y W N E T G F D L W E E V P G S S F F T I A S S H R
A L T E G A Y L A A Q L D T E C R A C T T V A P Q V L C F Q Q A F W N S K G N
Y V V S N I N G G E Y R S G K D A N S I L A S I H N F D P E A G C D N L T F Q
P C S E R A L A N H K A Y V D S F R N L Y A I N K G I A Q G K A V A V G R Y S
E D V Y Y N G N P W Y L A N F A A A E Q L Y D A I Y V W N K Q G S I T V T S V
S L P F F R D L V S S V S T G T Y S K S S S T F T N I V N A V K A Y A D G F I
E V A A K Y T P S N G A L A E Q Y D R N T G K P D S A A D L T W S Y S A F L S
A I D R R A G L V P P S W R A S V A K S Q L P S T C S R I E V A G T Y V A A T
S T S F P S K Q T P N P S A A P S P S P Y P T A C A D A S E V Y V T F N E R V
S T A W G E T I K V V G N V P A L G N W D T S K A V T L S A S G Y K S N D P L
W S I T V P I K A T G S A V Q Y K Y I K V G T N G K I T W E S D P N R S I T L
Q T A S S A G K C A A Q T V N D S W R

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Figure 3.



FIGURE 4A

AAGCTTACTAGTACTTCTCGAGCTCTGTACATGTCCGGTCGCGACGTACGCGTATCGATGGCGCCAGC  
TGCAGGCGGCCGCCTGCAGCCACTTGCAGTCCCGTGGAATTCTCACGGTGAATGTAGGCCTTTTGTAG  
GGTAGGAATTGTCACTCAAGCACCCCCAACCTCCATTACGCCTCCCCATAGAGTTCCCAATCAGTGA  
GTCATGGCACTGTTCTCAAATAGATTGGGGAGAAGTTGACTTCCGCCCAGAGCTGAAGGTCGCACAAC  
CGCATGATATAGGGTCGGCAACGGCAAAAAAGCACGTGGCTCACCGAAAAGCAAGATGTTTGCATCT  
AACATCCAGGAACCTGGATACATCCATCATCACGCACGACCACTTTGATCTGCTGGTAAACTCGTATT  
CGCCCTAAACCGAAGTGCCTGGTAAATCTACAGTGGGCCCCCTTTCGGTATACTGCGTGTCTTCTC  
TAGGTGCCATTCTTTTCCCTTCCTCTAGTGTGAATTGTTTGTGTGGAGTCCGAGCTGTAACCTACCT  
CTGAATCTCTGGAGAATGGTGGACTAACGACTACCGTGCACCTGCATCATGTATATAATAGTGATCCT  
GAGAAGGGGGGTTTGGAGCAATGTGGGACTTTGATGGTCATCAAACAAAGAACGAAGACGCCTCTTTT  
GCAAAGTTTTGTTTCCGCTACGGTGAAGAACTGGATACTTGTTGTGTCTTCTGTGTATTTTTGTGGCA  
ACAAGAGGCCAGAGACAATCTATTCAAACACCAAGCTTGCTCTTTTGAGCTACAAGAACCTGTGGGGT  
ATATATCTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGA  
AGCTGCTGCGAACCCGGAGAATCGAGATGTCTGGAAAGCTTCTAGCGAGCGGCTAAATTAGCATGAA  
AGGCTATGAGAAATTCTGGAGACGGCTTGTGAATCATGGCGTTCCATTCTTCGACAAGCAAAGCGTT  
CCGTGCGAGTAGCAGGCACTCATTTCCCGAAAAAACTCGGAGATTCCCTAAGTAGCGATGGAACCGGAAT  
AATATAATAGGCAATACATTGAGTTGCCTCGACGGTTGCAATGCAGGGGTACTGAGCTTGGACATAAC  
TGTTCCGTACCCCCACCTCTTCTCAACCTTTGGCGTTTCCCTGATTACGCGTACCCGTACAAGTCGTAA  
TCACTATTAACCCAGACTGACCGGACGTGTTTTGCCCTTCATTTGGAGAAATAATGTCATTGCGATGT  
GTAATTTGCCTGCTTGACCGACTGGGGCTGTTGCAAGCCCGAATGTAGGATTGTTATCCGAACCTCTGC  
TCGTAGAGGCATGTTGTGAATCTGTGTGCGGCAGGACACGCCTCGAAGGTTACGGCAAGGGAAACCA  
CCGATAGCAGTGTCTAGTAGCAACCTGTAAAGCCGCAATGCAGCATCACTGGAAAATACAAACCAATG  
GCTAAAAGTACATAAGTTAATGCCTAAAGAAGTCATATACCAGCGGCTAATAATTGTACAATCAAGTG  
GCTAAACGTACCGTAATTTGCCAACGGCTTGTGGGGTTGCAGAAGCAACGGCAAAGCCCCACTTCCCC  
ACGTTTGTCTTCACTCAGTCCAATCTCAGCTGGTGATCCCCCAATTGGGTCGCTTGTTTGTTCGG  
TGAAGTGAAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACAACCAAGGGCAGTGA  
TGGAAGACAGTGAAATGTTGACATTCAAGGAGTATTTAGCCAGGGATGCTTGAGTGATCGTGTAAAG  
AGGTTTGTCTGCCGATACGACGAATACTGTATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTA  
AAGTCGGCACTGAACAGGCAAAAGATTGAGTTGAAACTGCCTAAGATCTCGGGCCCTCGGGCCTTCGG  
CCTTTGGGTGTACATGTTTGTGCTCCGGGCAAATGCAAAGTGTGGTAGGATCGAACACACTGCTGCCT  
TTACCAAGCAGCTGAGGGTATGTGATAGGCAAATGTTTCAGGGGCCACTGCATGGTTTTGAAATAGAAAG  
AGAAGCTTAGCCAAGAACAATAGCCGATAAAGATAGCCTCATTAACCGGAATGAGCTAGTAG

FIGURE 4B

GCAAAGTCAGCGAATGTGTATATATAAAGGTTTCGAGGTCCGTGCCTCCCTCATGCTCTCCCCATCTAC  
TCATCAACTCAGATCCTCCAGGAGACTTGTACACCATCTTTTGAGGCACAGAAACCAATAGTCAACC  
ATCACAAGTTTGTACAAAAAGCAGGCTCCGCGGCCGCCCCCTTCAACATGCATACCTTCTCCAAGCT  
CCTCGTCTGGGCTCTGCCGTCCAGTCTGCCCTCGGGCGGCCTCACGGCTCTTCGCGTCTCCAGGAAC  
GCGTGGCGTTGATACCTTCATCAACACCGAGAAGCCCATCGCATGGAACAAGCTGCTCGCCAACATC  
GGCCCTAACGGCAAAGCCGCTCCCGGTGCCGCCGCGGCGTTGTGATTGCCAGCCCTTCAGGACGGA  
CCCTCCTTGTACGTGGTGGCATGGAATGGACCCAAGAGACTGGT'TTTAGATGAAAGAGAGTTTCTGCT  
AACCGCCACACCCAGACTTCTTCACCTGGACCCGCGATGCCGCCCTGGTCTCACCGGCATCATCGAG  
TCCCTTGGCCACAATAACAACACCACCTGACAGACCGTCATCCAGAACTACGTGCGTTCGAGGCCAA  
GCTGCAGCAGGTCTCGAACCCCTCGGGAACCTTCGCCGACGGCTCGGGTCTCGGTGAGGCCAAGTTCA  
ATGTCGACCTCACTGCCTTCACTGGCGAATGGGGTCGCCCTCAGAGGGACGGCCCGCCCTGCGCGCC  
ATCGTCTCATCCAGTACGCCAAGTGGCTGATCGCCAACGGCTACAAGAGCACGGCCAAGAGCGTCGT  
CTGGCCCGTCGTCAAGAACGATCTCGCCTACACGGCCAGTACTGGAACGAGACCGGCTTCGATCTCT  
GGGAGGAGGTCCCCGGCAGCTCGTTCCTTACCATCGCCAGCTCTCACAGGGGTGAGTCATTTATTGTT  
CAGTGTTTTCTCATTGAATAATTACCGAATGCCACTGACGCCAAACAGCTCTGACTGAGGGTGCTTA  
CCTCGCCGCTCAGCTCGACACCGAGTGCCGCGCCTGCACGACCGTCGCCCCTCAGGT'TCTGTGCTTCC  
AGCAGGCCCTTCTGGAACCTCAAGGGCAACTATGTCGTCTCCAACAGTAAGATCCCTACACCAACAAAA  
AAAATCGAAAAGGAACGTTAGCTGACCTTCTAGTCAACGGCGGCGAGTATCGCTCCGGCAAGGACGC  
CAACTCGATCCTGGCGTCCATCCACAAC'TTCGACCTGAGGCCGGCTGCGACAACCTGACCTTCCAGC  
CCTGCAGCGAGCGCGCCCTGGCCAACCACAAGGCCTATGTCGACTCGTTCCGCAACCTCTACGCCATC  
AACAAGGGCATCGCCAGGGCAAGGCCGTTGCCGTGCGCCGCTACTCGGAGGATGTCTACTACAACGG  
CAACCCGTGGTACCTGGCCAAC'TTGGCGCCGCGAGCAGCTCTACGACGCCATCTACGTGTGGAACA  
AGCAGGGCTCCATCACCGTGACCTCGGTCTCCCTGCCCTTCTTCCGCGACCTTGTCTCGTCGGTCAGC  
ACCGGCACCTACTCCAAGAGCAGCTCGACCTTACCAACATCGTCAACGCCGTCAAGGCCTACGCCGA  
CGGCTTCATCGAGGTGGCGGCCAAGTACACCCCGTCCAACGGCGCGCTCGCCGAGCAGTACGACCGCA  
ACACGGGCAAGCCCGACTCGGCCGCCGACCTGACGTGGTCTGTA'CTCGGCCTTCCTCTCGGCCATCGAC  
CGCCGCGCGGGTCTCGTCCCCCGAGCTGGCGGGCCAGCGTGGCCAAGAGCCAGCTGCCGTCCACCTG  
CTCGCGCATCGAGGTGCGCCGGCACCTACGTGCGCCGCCAGAGCACCTCGTTCCCGTCCAAGCAGACCC  
CGAACCCCTCCGCGGCGCCCTCCCGTCCCGCTACCCGACCGCCTGCGCGGACGCTAGCGAGGTGTAC  
GTCACCTTCAACGAGCGGTGTGACCGCGTGGGGCGAGACCATCAAGGTGGTGGGCAACGTGCCGGC  
GCTGGGGAAC'TGGGACACGTCCAAGGCGGTGACCCTGTGCGCCAGCGGGTACAAGTCGAATGATCCCC  
TCTGGAGCATCACGGTGCCCATCAAGGCGACGGGCTCGGCCGTGAGTACAAGTATATCAAG

FIGURE 4C

GTCGGCACCAACGGGAAGATTACTTGGGAGTCGGACCCCAACAGGAGCATTACCCTGCAGACGGCGTC  
GT  
CTGCGGGCAAGTGC GCCGCGCAGACGGTGAATGATTCGTGGCGTTAAAAGGGTGGGCGCGCCGACCCA  
GCTTTCCTGTACAAAGTGGTGATCGCGCCAGCTCCGTGCGAAAGCCTGACGCACCGGTAGATTCTTG  
TGAGCCCGTATCATGACGGCGGCGGGAGCTACATGGCCCCGGGTGATTATTTTTTTTGTATCTACTT  
CTGACCCTTTTCAAATATACGGTCAACTCATCTTCACTGGAGATGCGGCCTGCTTGGTATTGCGATG  
TTGTCAGCTTGGCAAATTGTGGCTTTCGAAAACACAAAACGATTCCCTTAGTAGCCATGCATTTTAAGA  
TAACGGAATAGAAGAAAGAGGAAATTAAAAAAAAAAAAAAAAACAAACATCCCGTTCATAACCCGTAGA  
ATCGCCGCTCTTCGTGTATCCCAGTACCAGTTTATTTTGAATAGCTCGCCCGCTGGAGAGCATCTGA  
ATGCAAGTAACAACCGTAGAGGCTGACACGGCAGGTGTTGCTAGGGAGCGTCGTGTTCTACAAGGCCA  
GACGTCTTCGCGGTTGATATATATGTATGTTTGA CTG CAGGCTGCTCAGCGACGACAGTCAAGTTCGC  
CCTCGCTGCTTGTGCAATAATCGCAGTGGGGAAGCCACACCGTGA CTCCCATCTTTCAGTAAAGCTCT  
GTTGGTGTTTATCAGCAATACACGTAATTTAAACTCGTTAGCATGGGGCTGATAGCTTAATTACCGTT  
TACCAGTGCCATGGTTCTGCAGCTTTCCTTGGCCCGTAAAATTGGGCGAAGCCAGCCAATCACCAGCT  
AGGCACCAGCTAAACCTATAATTAGTCTCTTATCAACACCATCCGCTCCCCCGGGATCAATGAGGAG  
AATGAGGGGGATGCGGGGCTAAAGAAGCCTACATAACCCTCATGCCAACTCCAGTTTACACTCGTCG  
AGCCAACATCTGACTATAAGCTAACACAGAATGCCCTCAATCCTGGGAAGAACTGGCCGCTGATAAGC  
GCGCCCGCCTCGCAAAAACCATCCCTGATGAATGGAAAGTCCAGACGCTGCCTGCGGAAGACAGCGTT  
ATTGATTTCCCAAAGAAATCGGGGATCCTTTTCAGAGCCGAACTGAAGATCACAGAGGCCTCCGCTGC  
AGATCTTGTGTCCAAGCTGGCGGCCGAGAGTTGACCTCGGTGGAAGTTACGCTAGCATTCTGTAAAC  
GGGCAGCAATCGCCAGCAGTTAGTAGGGTCCCCCTCTACCTCTCAGGGAGATGTAACAACGCCACCTT  
ATGGGACTATCAAGCTGACGCTGGCTTCTGTGCAGACAACTGCGCCACGAGTTCTTCCCTGACGCC  
GCTCTCGCGCAGGCAAGGGA ACTCGATGAATACTACGCAAAGCACAAGAGACCCGTTGGTCCACTCCA  
TGGCCTCCCCATCTCTCTCAAAGACCAGCTTCGAGTCAAGGTACACCGTTGCCCCCTAAGTCGTTAGAT  
GTCCCTTTTTGTGAGCTAACATATGCCACCAGGGCTACGAAACATCAATGGGCTACATCTCATGGCTA  
AACAAGTACGACGAAGGGGACTCGGTTCTGACAACCATGCTCCGCAAAGCCGGTGCCGTCTTCTACGT  
CAAGACCTCTGTCCCGCAGACCCTGATGGTCTGCGAGACAGTCAACAACATCATCGGGCGCACCGTCA  
ACCCACGCAACAAGAACTGGTCGTGCGGCGGCAGTTCTGGTGGTGAGGGTGCGATCGTTGGGATTGCG  
RVTGGTGGCGTCATCGGTGTAGGAACGGATATCGGTGGCTCGATTGAGTGCCGGCCCGGTTCAACTT  
CCTGTACGGTCTAAGGCCGAGTCATGGGCGGCTGCCGTATGCAAAGATGGCGAACAGCATGGAGGGTC  
AGGAGACGGTGACAGCGTTGTGCGGCCGATTACGCACTCTGTTGAGGGTGAGTCCTTCGCCTCTTCC  
TTCTTTTCTGCTCTATACCAGGCCTCCACTGTCTCTCTTTCTTGCTTTTATACTATATACGAGACC  
GGCAGTCACTGATGAAGTATGTTAGACCTCCGCCTCTTCACCAAATCCGTCCTCGGTCAG

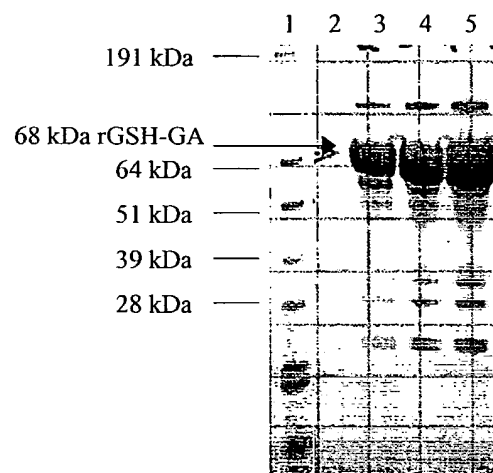
## FIGURE 4D

GAGCCATGGAAATACGACTCCAAGGTCATCCCCATGCCCTGGCGCCAGTCCGAGTCGGACATTATTGCT  
CT  
CCAAGATCAAGAACGGCGGGCTCAATATCGGCTACTACAACCTCGACGGCAATGTCCTTCCACACCCT  
CCTATCCTGCGCGGCGTGGAAACCACCGTCGCCGCACTCGCCAAAGCCGGTCACACCGTGACCCCGTG  
GACGCCATACAAGCACGATTTTCGGCCACGATCTCATCTCCCATATCTACGCGGCTGACGGCAGC<sub>RVG</sub>  
CCGACGTAATGCGCGATATCAGTGCATCCGGCGAGCCGGCGATTCCAAATATCAAAGACCTACTGAAC  
CCGAACATCAAAGCTGTTAACATGAACGAGCTCTGGGACACGCATCTCCAGAAGTGGAATTACCAGAT  
GGAGTACCTTGAGAAATGGCGGGAGGCTGAAGAAAAGGCCGGGAAGGAACTGGACGCCATCATCGCGC  
CGATTACGCCTACCGCTGCGGTACGGCATGACCAGTTCGGTACTATGGGTATGCCTCTGTGATCAAC  
CTGCTGGATTTACGAGCGTGGTTGTTCCGGTTACCTTTGCGGATAAGAACATCGATAAGAAGAATGA  
GAGTTTCAAGGCGGTTAGTGAGCTTGATGCCCTCGTGAGGAAGAGTATGATCCGGAGGCGTACCATG  
GGGCACCGGTTGCAGTGCAGGTTATCGACGGAGACTCAGTGAAGAGAGGACGTTGGCGATTGCAGAG  
GAAGTGGGGAAGTTGCTGGGAAATGTGGTGACTCCATAGCTAATAAGTGTGAGATAGCAATTTGCACA  
AGAAATCAATACCAGCAACTGTAAATAAGCGCTGAAGTGACCATGCCATGCTACGAAAGAGCAGAAAA  
AAACCTGCCGTAGAACCGAAGAGATATGACACGCTTCCATCTCTCAAAGGAAGAATCCCTTCAGGGTT  
GCGTTTCCAGTCTAGACACGTATAACGGCACAAGTGTCTCTACCAAATGGGTTATATCTCAAATGTG  
ATCTAAGGATGGAAAGCCCAGAATATCGATCGCGCGAGATCCATATATAGGGCCCGGGTTATAATTA  
CCTCAGGTCGACGTCCCATGGCCATTGCAATTCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATT  
GTTATCCGCTCACAATTCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCATA  
TGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCCGGAAACCTGTCGTG  
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAG  
AGGCGGTTTTCGCTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCTTCGGC  
TGCGGCGAGCGGTATCAGCTCACTCAAAGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA  
GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT  
TTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACC  
CGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCTGTTCCGACC  
CTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTCTCATAGCTCAGG  
CTGTAGGTATCTCAGTTCGGTGTAGGTGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTT  
AGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGTAAGACACGACTTATCG  
CCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTT  
GAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAG  
TTACCTTCGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTT  
TTTGTTCGAAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTAC  
GGGGTCTGACGCTCAGTGGAACGA

FIGURE 4E

AAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATT  
AAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTA  
ATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGT  
GTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCAC  
GCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCT  
GCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCGGGAAGCTAGAGTAAGTAGTTCGCCAGT  
TAATAGTTTGC GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGG  
CTTCATTACAGCTCCGGTTCCTAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAGCG  
GTTAGCTCCTTCGGTCTCCGATCGTTGTGAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTAT  
GGCAGCACTGCATAATTCTCTTACTGTGATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT  
CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGAT  
AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGAAAACGTTCTTCGGGGCGAAAAC  
CTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGACCCAACTGATCTTCAG  
CATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGA  
ATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATTATCA  
GGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCGCG  
GCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATATTATCATGACATTAACCTATAAA  
AATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACAT  
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCG  
CGTCAGCGGTGTTGGCGGGTGTGCGGGCTGGCTTAACATATGCGGCATCAGAGCAGATTGTACTGAGA  
GTGCACCATAAAATTGTAAACGTTAATATTTTGTAAATTCGCGTTAAATTTTGTAAATCAGCTC  
ATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCGAGATAGGGT  
TGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGA  
AAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCAAATCAAGTTTTTTGGGGTCGAG  
GTGCCGTAAAGCACTAAATCGGAACCTAAAGGGAGCCCCGATTTAGAGCTTGACGGGGAAAGCCGG  
CGAACGTGGCGAGAAAGGAAGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG  
GTCACGCTGCGCGTAACCAACACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTACTATGGTTG  
CTTTGACGTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCATTC  
GCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGG  
CGAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTGT  
AAAACGACGGCCAGTGCCC

Figure 5 *T. reesei* expressing  
recombinant *H. grisea* GSH-GA



Lane 1. SeeBlue Marker  
Lane 2. Blank  
Lane 3. 48 hr sample  
Lane 4. 56 hr sample  
Lane 5. 64 hr sample

FIGURE 6: Genomic Nucleotide Sequence of *Aspergillus kawachi*  
GSHE

ATGTCGTTCCGATCTCTTCTCGCCCTGAGCGGCCTTGTCTGCTCGGGGTGGCAAGTGTGAT  
TTCCAAGCGCGCGACCTTGGATTCTGTTGAGCAACGAAGCGACCGTGGCCCGTACTGCGA  
TCCTGAATAACATCGGGGCGGACGGTGCTTGGGTGTCGGGCGCGGACTCTGGCATTGTCTGT  
GCCAGTCCCAGCACCAGATAACCCGACTGTATGTTTTGAGTTCGGATTATGAATGTGTCTTG  
GTTGATTGATGCTGACTGGCGTGTCTTTTGATGATTGTAGACTTCTACACCTGGACTCGCGA  
CTCTGGTCTCGTCATCAAGACCCTCGTCGACCTCTTCCGCAATGGAGATACTGATCTCCTTT  
CCACCATTGAGCACTACATCTCCTCTCAGGCAATTATTCAGGGTGTGAGTAACCCCTCTGGT  
GATCTGTCCAGCGGTGGTCTTGGTGAGCCCAAGTTCAATGTCGATGAGACTGCCTACACCGG  
TTCTTGGGGACGGCCGAGCGTGATGGTCCTGCCCTGAGAGCAACTGCTATGATCGGCTTTG  
GGCAGTGGCTGCTTGTATGTTCTCCACCTCCTTGCGTCTGATCTGCAACATATGTAGCCGAC  
TGGTCAGGACAATGGCTACACCAGCGTGCAACAGAGATTGTTTGGCCCTCGTTAGGAACG  
ACCTGTCTGATGTGGCTCAGTACTGGAACCAGACGGGATATGGTGTGTTTGATTGATCGGGG  
TTCAAGGGTGTGTTGTGCATCGGAGCTAACTTCGCGGTGCGAGATCTCTGGGAAGAAGTTAAT  
GGCTCGTCCTTCTTCACTATTGCCGTGCAACACCGCGCCCTCGTCGAAGGTAGTGCCTTCGC  
GACGGCCGTGGCTCGTCCTGCTCCTGGTGTGATTGCGAGGCACCTCAGATTCTCTGTTACT  
TGCACTCCTTCTGGACCGGCAGCTACATCCTGGCCAACCTTGACAGCAGCCGTTCCGGCAAG  
GACACAAACACCCTCCTGGGAAGCATCCACACCTTTGATCCTGAGGCTGGATGCGACGACTC  
CACCTTCCAGCCCTGCTCCCCGCTGCGCTCGCCAACCATAAGGAGGTGTAGACTCTTTCC  
GCTCGATCTATACTCTCAACGATGGTCTCAGTGACAGTGAGGCGGTGCGGTGCGGTAC  
CCTGAGGATAGCTACTACAACGGCAACCCGTGGTTCCTGTGCACCTTGGCTGCCGCGGAACA  
GCTGTACGATGCTCTGTACCAGTGGGACAAGCAGGGGTGCTTGGAGATCACAGACGTGTCAC  
TTGACTTCTTCAAGGCTCTGTACAGTGGTGCTGCCACCGGCACGTACTCTTCGTCCAGCTCG  
ACCTATAGCAGCATTGTGAGTGCCGTCAAGACTTTGCTGATGGTTTTGTCTTATTGTGGT  
AAGTCTACGCTAGACGAGCGCTCATATTTACAGAGGGTGCCTACTAACAGGATTAGGAACT  
CACGCCGCAAGCAACGGCTCTCTGTCTGAGCAATTCGACAAGTCTGATGGCGACGAGCTTTC  
TGCTCGCGATCTGACCTGGTCTTACGCTGCTCTGCTGACCGCCAACAACCGTCGTAATTCTG  
TCGTGCCCCCGTCTTGGGGTGAGACCTCTGCCAGCAGCGTGCCCGCACCTGTGCGGTACC  
TCTGCCCTCTGGTACCTACAGCAGTGTGACCGTCACCTCGTGGCCGAGCATCGTGGCTACTGG  
TGGCACCCTACGACGGCTACTACCACTGGATCGGGCGGCGTGACCTCGACCAGCAAGACCA  
CCACAACCTGCTAGTAAGACCAGCACCCTACGTCCTCGACCTCCTGCACCACCCCACTGCC  
GTAGCTGTGACCTTTGATCTGACGGCGACCACCACTACGGCGAGAACATCTACCTGGTCCG  
GTCGATCTCTCAGCTCGGTGACTGGGAGACCAGCGATGGCATAGCTCTGAGCGCTGACAAGT  
ACACTTCCAGCAACCCGCTTTGGTATGTAACGTGACTCTGCCGGCTGGTGAGTCATTTGAG  
TACAAGTTCATCCGCGTCGAGAGCGATGACTCCGTGGAGTGGGAGAGCGACCCGAACCGGGA  
ATACACCGTTCCTCAGGCGTGCGGCGAGTCGACCGCGACGGTGACCGACACCTGGCGGTAG



FIGURE 7A: *Aspergillus awamori* var. *kawachi* GSHE precursor (i.e.: with signal sequence underlined) protein sequence.

M S F R S L L A L S G L V C S G L A S V I S K R A T L D S W L  
S N E A T V A R T A I L N N I G A D G A W V S G A D S G I V V  
A S P S T D N P D Y F Y T W T R D S G L V I K T L V D L F R N  
G D T D L L S T I E H Y I S S Q A I I Q G V S N P S G D L S S  
G G L G E P K F N V D E T A Y T G S W G R P Q R D G P A L R A  
T A M I G F G Q W L L D N G Y T S A A T E I V W P L V R N D L  
S Y V A Q Y W N Q T G Y D L W E E V N G S S F F T I A V Q H R  
A L V E G S A F A T A V G S S C S W C D S Q A P Q I L C Y L Q  
S F W T G S Y I L A N F D S S R S G K D T N T L L G S I H T F  
D P E A G C D D S T F Q P C S P R A L A N H K E V V D S F R S  
I Y T L N D G L S D S E A V A V G R Y P E D S Y Y N G N P W F  
L C T L A A A E Q L Y D A L Y Q W D K Q G S L E I T D V S L D  
F F K A L Y S G A A T G T Y S S S S S T Y S S I V S A V K T F  
A D G F V S I V E T H A A S N G S L S E Q F D K S D G D E L S  
A R D L T W S Y A A L L T A N N R R N S V V P P S W G E T S A  
S S V P G T C A A T S A S G T Y S S V T V T S W P S I V A T G  
G T T T T A T T T G S G G V T S T S K T T T T A S K T S T T T  
S S T S C T T P T A V A V T F D L T A T T T Y G E N I Y L V G  
S I S Q L G D W E T S D G I A L S A D K Y T S S N P L W Y V T  
V T L P A G E S F E Y K F I R V E S D D S V E W E S D P N R E  
Y T V P Q A C G E S T A T V T D T W R

FIGURE 7B: Mature (i.e. expressed protein with the putative signal sequence removed) *Aspergillus awamori* var. *kawachi* GSHE protein sequence.

ATLDSWLSNEATVARTAILNIGADGAWVSGADSGIVVASPSTDNPDYFYTWTRDSGLVIKT  
LVDLFRNGD TDLLSTIEHYISSQAI IQGVSNPSGDLSSGGLGEPKFNVDE TAYTGSWGRPQR  
DGPALRATAMIGFGQWLLDNGY TSAATEIVWPLVRNDLSYVAQYWNQTGYDLWEEVNGSSFF  
TIAVQHRALVEGSAFATAVGSSCSWCDSQAPQILCYLQSFWTGSYILANFDSSRSGKDTNTL  
LGSIH TFDPEAGCDDSTFQPCSPRALANHKEVVD SFRSIYTLNDGLSDSEAVAVGRYPEDSY  
YNGNPWF LCTLA AAEQLYDALYQWDKQGSLEITDVSLDFFKALYSGAATGTYS SSSSTYSSI  
VSAVKTFADGFVSIVETHAASNGSLSEQFDKSDGDELSARDLTWSYAALLTANNRRNSVVP  
SWGETSASSVPGTCAATSASGTYS SVTVTSWPSIVATGGTTTTATTTGSGGVTSTSKTTTTA  
SKTSTTTSSTCTTPTAVAVTFDLTATTTYGENIYLVGSISQLGDWETSDGIALSADKYTSS  
NPLWYVTVTLPAGESFEYKFIRVESDDSV EWESDPNREYTVPQACGESTATVTD TWR

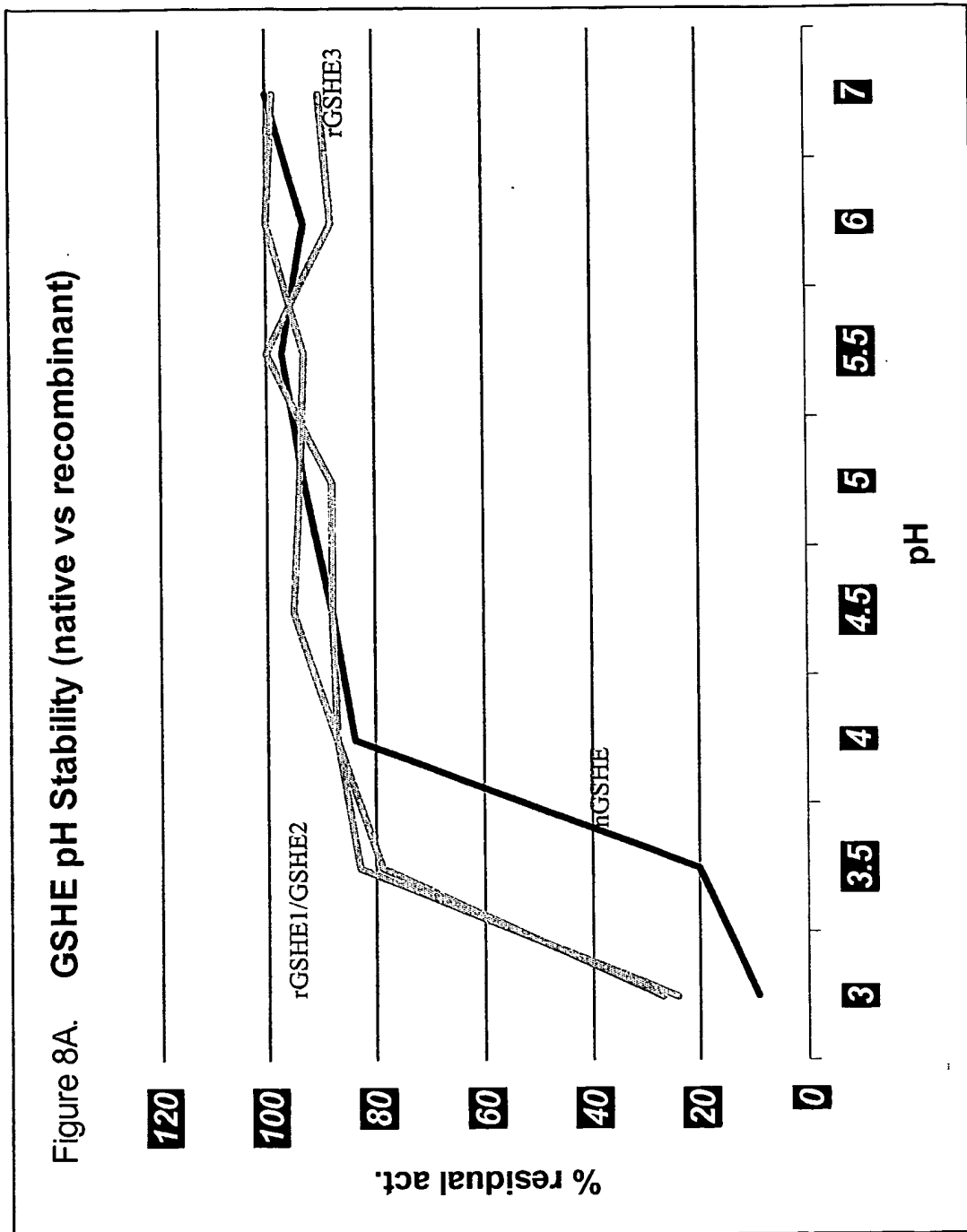


Figure 8B. GSHE pH Stability (native vs recombinant)

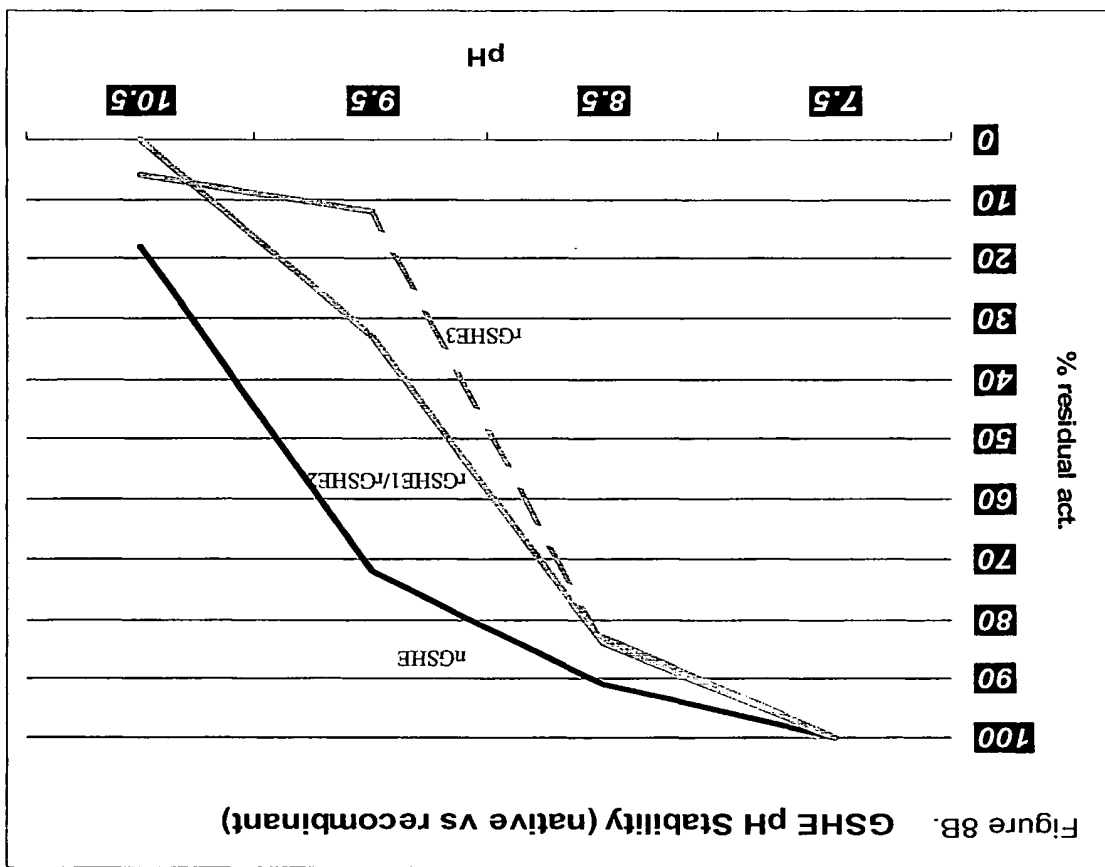
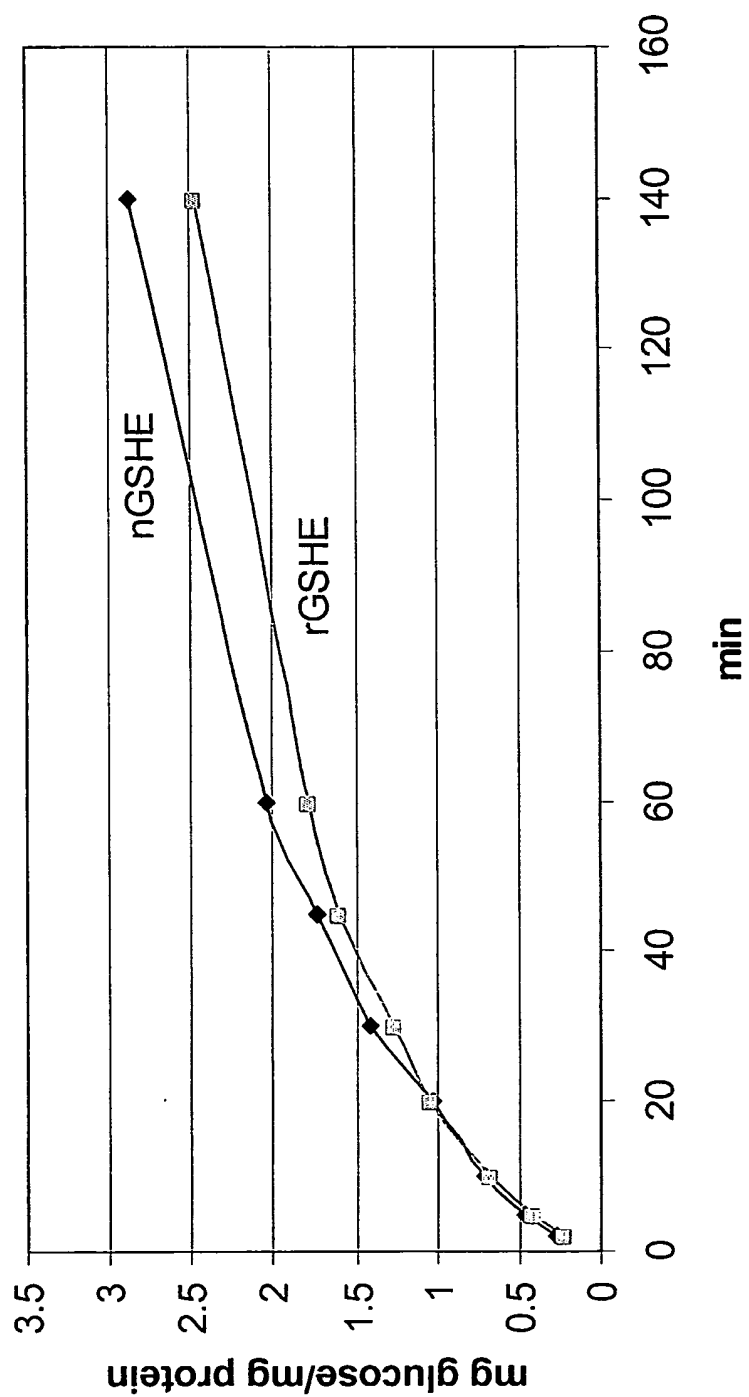


Figure 9. GSHE Starch Hydrolysis



*T. reesei* expressed *A. kawachi* GSHE

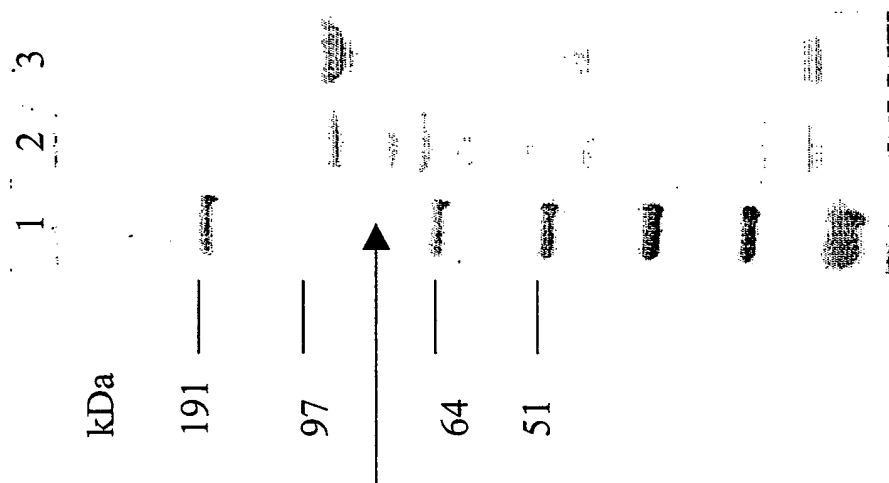


FIG. 10

FIG. 11

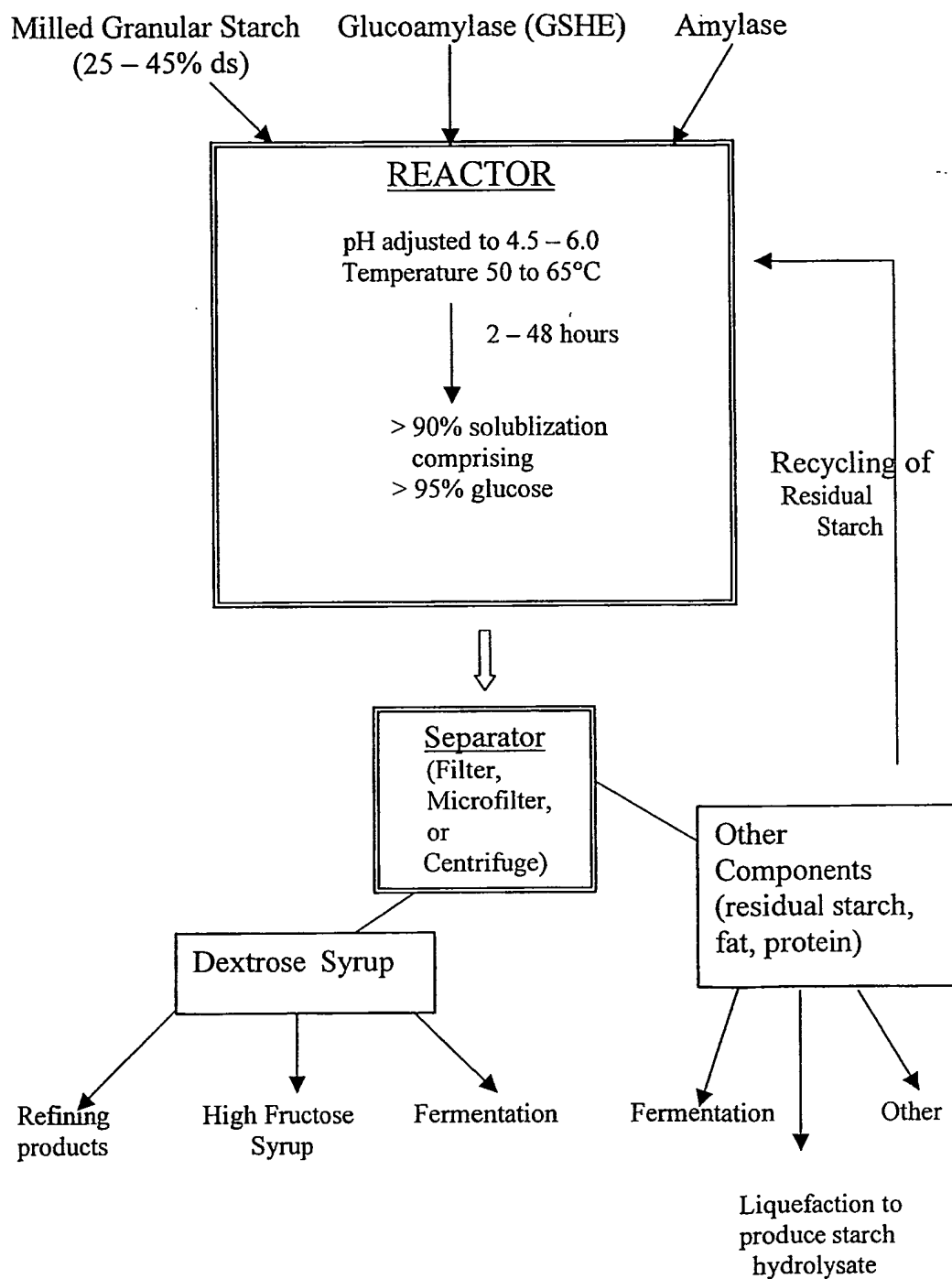
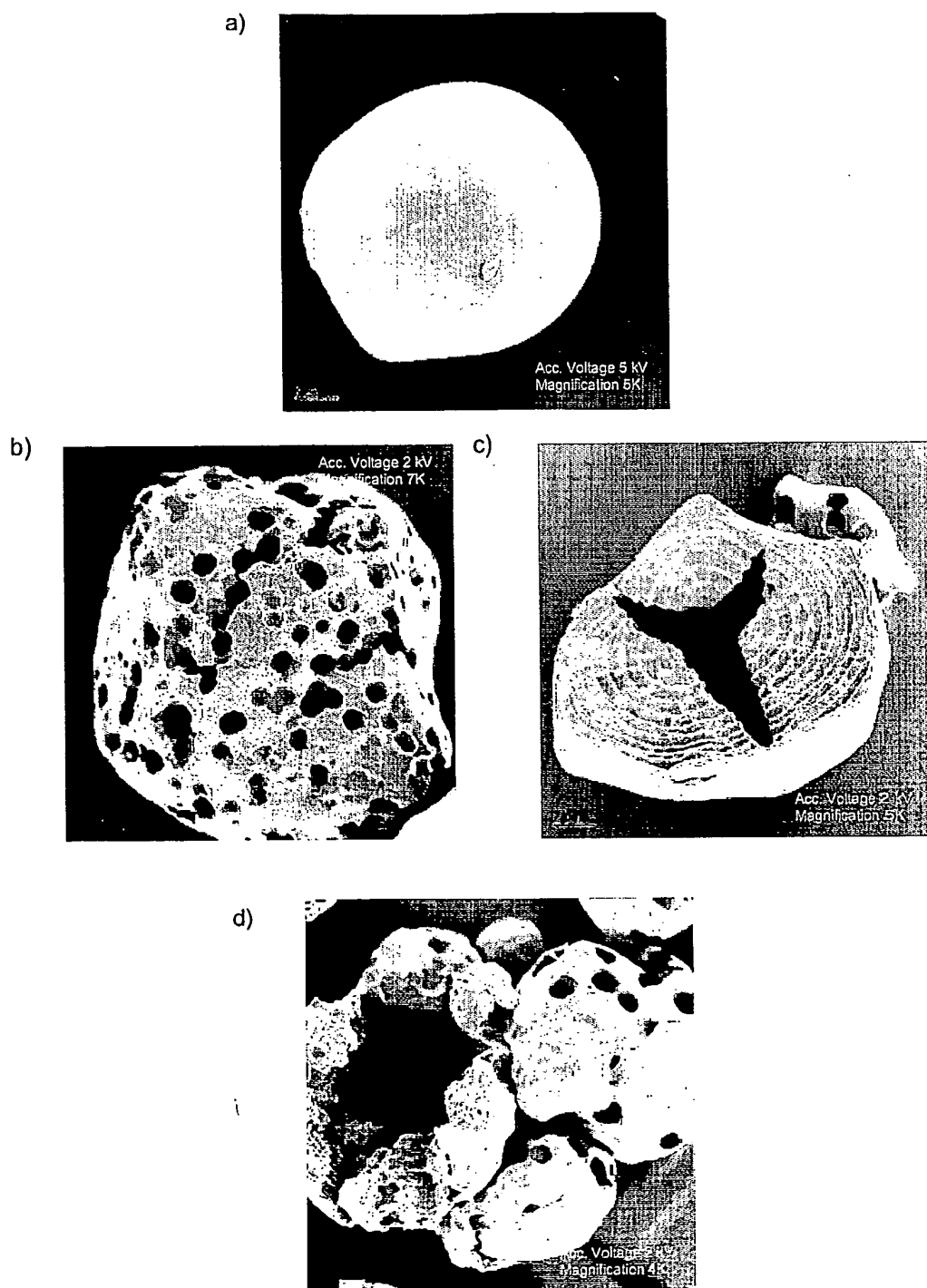


Figure 12





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